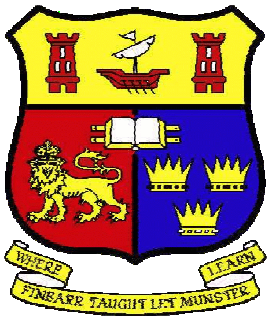
A study of diet and health in the elderly; the gut microbiota as a source of bioactive agents



A thesis presented to the National University of Ireland for the degree of

Doctor of Philosophy

by

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Dedicated to my parents, John and Eleanor Power

Declaration

I hereby declare that the content of this thesis is the result of my own work and has not been submitted for another degree either at University College Cork or elsewhere.

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Table of Contents

Contents ..... **i**

Abstract ..... **viii**

Preface ..... **xii**

Literature Review

Chapter 1 Intestinal microbiota, diet and health 1

Chapter 2 Intestinal microbiota, obesity and metabolic syndrome 47

Experimental Chapters

Chapter 3 Food and nutrient intake of Irish community-dwelling elderly subjects: who is at nutritional risk? 77

Chapter 4 Cognitive decline in non-diabetic elderly subjects associated with high glycaemic diet 113

Chapter 5 Immunomodulatory effects of 'viable' and 'non-viable' *Bifidobacterium*-fermented milks: IL-10 and TNF-α secretion by human peripheral blood mononuclear cells 139

Chapter 6 Antimicrobial production by intestinal bacteria from elderly subjects 175

Chapter 7 Draft genome sequence of *Lactobacillus crispatus* EM-LC1, an isolate with antimicrobial activity cultured from an elderly subject 217

General Discussion **253**

Appendices **262**

Acknowledgements **273**

List of Abbreviations

AAD	Antibiotic-associated diarrhoea
ABC	ATP-binding cassette
ACC	Acetyl-coA carboxylase
ACT	Artemis comparison tool
AIEC	adherent-invasive *E. coli*
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
APC	Antigen presenting cells/Alimentary Pharmabiotic Centre
BEA	Bile esculin agar
BF	Burkino Faso
BFM	*Bifidobacterium*-fermented milk
BHI	Brain heart infusion
BLAST	Basic local alignment search tool
BMI	Body mass index
BP	Blood pressure
CAZymes	Carbohydrate-active enzymes
CD	Crohn's disease
CD14	Cluster of differentiation 14
CDS	Coding DNA sequences
CFS	Cell-free supernatant
CFU	Colony forming units
CHEF	Clamped homogeneous electrophoresis field
CLA	Conjugated linoleic acid
COG	Clusters of orthologous proteins
CONV	Conventional
CRISPR	Clustered regularly interspaced short palindromic repeats
CT	Controlled trial
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DC	Dendritic cells
DGGE	Denaturing gradient gel electrophoresis
DHA	Docosahexaenoic acid
DIO	Diet-induced obesity
DMEM	Dulbecco's Minimum Essential Medium
DPC	Dairy Products Research Centre
e^{RC}	Exponential function of the regression coefficient
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
EPIC	European Prospective Investigation into Cancer
EPS	Exopolysaccharide
EU	European
FAS	Fatty acid synthase
FFQ	Food frequency questionnaire
fiaf	Fasting-inducted adipocyte factor
FISH	Fluorescent *in situ* hybridisation
GF	Germ-free
GFD	Gluten-free diet

GI	Gastro-intestinal/glycaemic index
GIT	Gastro-intestinal tract
GL	Glycaemic load
Glp	Glucagon-like peptide
GOLD	Genomes online database
GPR	G protein-coupled receptor
HDL	High-density lipoprotein
HFD	Healthy food diversity
HOMA	Homeostatic model assessment
HC	Healthy control
HP	High pressure
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IEC	Intestinal epithelial cell
IFN	Interferon
IL	Interleukin
KAAS	KEGG automatic annotation server
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAB	Lactic acid bacteria
LDL	Low-density lipoprotein
LGG	*Lactobacillus rhamnosus* GG
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
MCL	Markov clustering algorithm
mMRS	modified MRS
MMSE	Mini-Mental State Examination
MNA	Mini-nutritional assessment
MPa	Mega Pascal
MRD	Maximum recovery diluent
MRS	De Man Rogosa Sharpe
MSD	Meso Scale Discovery
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NAT	WC + N-S supplement
NAV	WC + G-N supplement
NCBI	National Centre for Biotechnology Information
NEC	Necrotizing enterocolitis
NKT	Natural killer T
NSP	Non-starch polysaccharide
ob/ob	Genetically obese mice
ORF	open reading frame
OTU	Operational taxanomic unit
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
PPAR	Peroxisomal proliferator-activated receptor
PTS	Phosphotransferase system
PYY	Peptide YY

qPCR	Quantitative polymerase chain reaction
RAPD PCR	Random amplified polymorphic DNA PCR
RC	Regression coefficient
RCA	Reinforced Clostridial Agar
RCT	Randomised controlled trial
rDNA	Ribosomal DNA
RCT	Randomised controlled trial
RELM-β	Resistin-like molecule β
R/M	Restriction/Modification
rRNA	Ribosomal RNA
SBP	Systolic blood pressure
SCD-1	Stearoyl-CoA desaturase
SCFA	Short chain fatty acid
SCID	Severe combined immunodeficient
SD	Standard deviation
sICAM-1	Soluble intercellular adhesion molecule-1
SLÁN	Survey of lifestyles attitudes and nutrition
T2D	Type 2 diabetes mellitus
T5KO	TLR-5–knock-out
TA	Toxin/antitoxin
TG	Triglyceride
TJ	Tight junction
TLR	Toll-like receptor
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
Tn	Naive T cell
TNF	Tumor necrosis factor
Treg	Regulatory T cell
tRNA	Transfer RNA
UC	Ulcerative colitis
UCP-2	Uncoupling protein-2
VLDL	Very low-density lipoprotein
VRB	Violet red bile
VRBD	Violet red bile dextrose
WC	Wilkins Chalgren

List of Publications

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Power SE, Fitzgerald GF, O'Toole PW, Ross RP, Stanton C, Quigley EMM and Murphy EF (2013) Metabolic Syndrome and Obesity in Adults. In *Probiotics in the Prevention and Treatment of Diseases in Adults and Children*, pp 103–121 [Guarino A, Quigley EE and Walker AW, editors]. *World Review of Nutrition and Dietetics* **107,** Basel: Karger DOI: 10.1159/000345750 **(Appendix 2).**

Claesson MJ, Jeffery IB, Conde S, **Power SE**, O'Connor EM, Cusack S, Harris HMB, Coakley M, Lakshminarayanan B, O'Sullivan O, Fitzgerald GF, Deane J, O'Connor M, Harnedy N, O'Connor K, O'Mahony D, van Sinderen D, Wallace M, Brennan L, Stanton C, Marchesi JR, Fitzgerald AP, Shanahan F, Hill C, Ross RP and O'Toole PW (2012) Gut microbiota composition correlates with diet and health in the elderly. *Nature* **488,** 178-184 **(Appendix 3).**

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Power SE, Harris HMB, Bottacini F, Ross RP, O'Toole PW and Fitzgerald GF (2013) Draft genome sequence of *Lactobacillus crispatus* EM-LC1, an isolate with antimicrobial activity cultured from an elderly subject (accepted for publication in *Genome Announcements*).

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Power SE, O'Toole PW, O'Connor EM and Fitzgerald GF (2012) Compliance with Irish food-based dietary guidelines in elderly subjects recruited from rehabilitation wards and out-patient clinics in Southern region hospitals (The ELDERMET project). *Proceedings of the Nutrition Society, Belfast, Ireland, 16th – 19th July 2012* **(Appendix 4).**

Power SE, O'Connor EM, Jeffery IB, O'Toole PW, and Fitzgerald GF (2012) Gender differences in food intake among Irish community-dwelling elderly subjects: The ELDERMET project. *Proceedings of the Nutrition Society, Belfast, Ireland, 16th – 19th July 2012* **(Appendix 5).**

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Abstract

The proportion of the global population that falls into the 'elderly' category is increasing rapidly. Poor dietary habits and inadequate nutrient intakes are of particular concern in this population group and may influence quality of life and health outcomes. The dietary intake of 208 Irish community-dwelling elderly individuals (64-93 yrs), participating in the ELDERMET project was assessed using a validated semi-quantitative food frequency questionnaire (FFQ). Consumption of energy-dense, low-nutrient foods was shown to be excessive. Older elderly subjects (≥75 yrs) consumed significantly more desserts/sweets than younger elderly (64-74 yrs). Intakes of dietary fat and saturated fat were high while dairy food consumption was inadequate in both males and females. Elderly females typically had a more nutrient dense diet than males and a considerable proportion of subjects, particularly males, had inadequate intakes of calcium, magnesium, vitamin D, folate, zinc and vitamin C. These findings highlight the need for appropriate interventions to tackle these dietary inadequacies in this vulnerable population group.

Additional analysis of this elderly cohort was undertaken in order to investigate the association between dietary patterns, dietary glycaemic index and glycaemic load, and cognition. Hierarchical clustering was conducted on the FFQs to derive patterns of dietary intake. Elderly subjects consuming 'prudent' dietary patterns (high in fruit, vegetables, and low in red meat and white bread) had better cognition function (as assessed by the Mini-mental State Examination) compared to those consuming 'Western' dietary patterns (high in red meat, white bread and low in fruit and vegetables). Furthermore, in fully adjusted models, logistic and Poisson regression analysis revealed that the high glycaemic diets were associated with poor cognitive function, demonstrating a new link between nutrition and cognition in the elderly.

A collaborative project was undertaken with Alimentary Health Ltd., to investigate if *Bifidobacterium*-fermented milk products (BFMs) containing non-viable ('dead') cells could stimulate levels of IL-10 and TNF-α in a manner comparable to those stimulated by BFMs containing viable ('live') cells. Depending on the strain and mode of killing used, BFMs containing non-viable cells stimulated levels of IL-10 and TNF-α which were comparable to those stimulated by BFMs containing viable cells in the peripheral blood mononuclear cell model. In particular, killing the cells by storage at 4ºC over a period of 9 weeks was found to be the best method for maintaining immunomodulatory

activity for all *Bifidobacterium* strains. As well as eliminating shelf life problems, administration of such a product containing non-viable microorganisms could reduce the risk of microbial translocation and infection. Nonetheless, the true *in vivo* picture needs to be fully determined.

An extensive screening study of the elderly faecal-derived microbiota was undertaken to examine the prevalence of antimicrobial production by intestinal bacteria and to mine this rich repository of metabolites for novel antimicrobials. Screening with the indicator organisms *Lb. delbrueckii* subsp. *bulgaricus* LMG6901 and *Escherichia coli* K-12 resulted in the isolation of 16 genetically distinct strains producing antimicrobial substances. A number of previously characterised bacteriocins including ABP-118 (from *Lactobacillus salivarius),* gassericin T *(Lactobacillus gasseri),* mutacin II *(Streptococcus mutans),* enterocin L-50 and enterocin P (*Enterococcus faecium)* were identified. A number of *E. coli* strains were also isolated which produced heat labile antimicrobials, most likely colicins or microcins. Interestingly, a *Lactobacillus crispatus* strain (EM-LC1) was isolated which was found to produce a potentially novel heat labile antimicrobial compound which was resistant to protease activity in the cell-free supernatant.

A genome sequencing approach was applied to attempt to identify loci which may be responsible for what initially appeared to be an atypical bacteriocin-like antimicrobial activity in the faecal isolate, *Lb. crispatus* EM-LC1. The draft genome sequence consists of 1,862,161 bp with a G+C content of 36.9%. It is predicted to contain 1,827 putative coding DNA sequences (CDSs) representing an 87.6% coding density. A predicted exopolysaccharide biosynthetic cluster, a complete type I restriction/modification system, a complete toxin-antitoxin system and individual phage-like genes were identified. As expected, a number of genes related to carbohydrate utilisation and genes implicated in adhesion to human intestinal cells were identified and are likely to contribute to the ability of *Lb. crispatus* to colonize intestinal mucosa. Comparison of the genome to the published genome sequence *Lb. crispatus* ST1 (which does not exhibit antimicrobial activity) revealed a number of genes related to bacteriocin (helveticin) production. Indeed, the genome sequence of this strain will offer a clearer genomic platform for further investigation into *Lb. crispatus*

antimicrobial activity and will elucidate the genetic basis for its potential probiotic traits.

Preface

As a society we are facing significant health problems. The incidence of chronic diseases, including obesity, type 2 diabetes mellitus, cardiovascular disease and cancer, is increasing worldwide and places a significant burden on national health budgets. Diet and nutrition have been recognised for many years as important factors in the promotion and maintenance of good health throughout life and their role as determinants of chronic disease is well established. Indeed, food provides nutrients needed to fuel, build and maintain all body cells and over- or under-consumption of particular nutrients can lead to an increased risk of diet-related disease.

Recent years have witnessed the increasing prominence of the gut microbiota as a major research topic in relation to health and disease, presenting new opportunities for medical and food applications. Indeed, while we have known for many years that certain microbial entities can cause diseases such as gastroenteritis, we are only starting to appreciate the more subtle role of the commensal intestinal microbiota in various intestinal conditions, such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) (Clemente *et al.,* 2012; Power *et al.,* 2013b) and even conditions beyond the gut, such as obesity and metabolic syndrome (Power *et al.,* 2013a)**.**

While understanding the role of the gut microbiota in health and disease is one of the very topical areas in science, mechanisms to manipulate the microbiota in order to generate a 'healthy' microbiota are an obvious requirement. There are several approaches to modulate the gut microbiota. The administration of antibiotics has long been recognised as having a major, and possibly negative impact on the balance of the microbiota, by reducing or removing specific bacterial species or classes (Claesson *et al.,* 2011; Young & Schmidt, 2004). In contrast, certain beneficial bacteria may be increased by the consumption of probiotics: '*live microorganisms which when administered in adequate amounts confer health benefits to the host*' (FAO/WHO, 2002). Although one of the original concepts associated with probiotics was that their consumption would alter the composition of the intestinal microbiota (i.e. replace 'bad' bacteria with 'good' bacteria) (Metchnikoff, 1908), it has since been suggested that too much emphasis should not be placed on the potential change in microbiota composition without bearing in mind any benefits to health that are accomplished (Ouwehand *et al.,* 2002). Indeed, for some probiotic health effects (e.g. immune modulation) it may not be necessary to achieve any measurable modification of the intestinal microbiota

composition. There is currently a vast array of proposed health effects (both direct and indirect) attributed to specific probiotics (Goldin, 2011) which has provided major commercial opportunities for a number of food companies worldwide, with a global market estimated at $24.2 billion in 2011(MarketsandMarkets, 2013)

More recently, diet has been highlighted as one of the key methods for altering the microbiota (Claesson *et al.,* 2012; De Filippo *et al.,* 2010; Wu *et al.,* 2011). Diet provides nutrients not only for the host but also for the bacteria in the GIT. The ELDERMET project has provided ground-breaking evidence which supports the link between diet, the gut microbiota and health in the elderly and suggests that changing the diet to drive a beneficial change in the microbiota composition could support better health and healthier ageing in the older population (Claesson *et al.,* 2012). These findings represent opportunities for both the food ingredients business and the pharmaceutical industry to develop specific food ingredients that promote healthier ageing, through modification of the microbiota composition.

Another element in the study of the intestinal microbiota is the realisation that the microbiota can be a source of valuable functional compounds with potential biomedical applications (such as bacteriocins, immunomodulatory components and bacteriophages). Indeed, ongoing 'mining' of the gastrointestinal microbiota by scientists in the Alimentary Pharmabiotic Centre (APC) has shown it to be a rich repository of functional food ingredients and even new drugs. For example, the APC scientists have discovered, patented and licensed a narrow-spectrum antimicrobial active against *Clostridium difficile,* which may offer an alternative treatment for *C. difficile*-associated diarrhoea (Rea *et al.,* 2010).

This current project was supported by the ELDERMET project, the APC and by the Irish Research Council under the Enterprise Partnership Scheme (in collaboration with the industry partner, Alimentary Health Ltd.). The overall hypothesis of this thesis is that diet and the intestinal microbiota independently and jointly contribute to health in the elderly and that the investigation of habitual dietary patterns and functional microbiota components, can lead to the identification of specific effector mechanisms. The initial phase of this project investigated the dietary intake of elderly subjects participating in the ELDERMET project (Chapter 3) which provided the opportunity to make associations with cognitive health (Chapter 4). Following on from this, a

collaborative project with Alimentary Health Ltd. allowed the investigation of the immunomodulatory effect of 'live' and 'dead' *Bifidobacterium*-fermented milk products in a cell type representative of the innate immune system (Chapter 5). Lastly, this project included an extensive screening study of the elderly-derived faecal microbiota in order to examine the prevalence of antimicrobial production by intestinal bacteria and to mine this rich repository of metabolites for novel antimicrobials (Chapter 6). This was followed by the determination of the genome sequence of *Lb. crispatus* EM-LC1 (Chapter 7), an isolate selected for its potentially novel antimicrobial activity (Chapter 6).

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CHAPTER I

Intestinal microbiota, diet and health

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1.0 Abstract

The human intestine is colonised by 10^{13} to 10^{14} microorganisms, the vast majority of which belong to the phyla of *Firmicutes* and *Bacteroidetes*. Although highly stable over time, the composition and activities of the microbiota may be influenced by a number of factors including age, diet, and antibiotic treatment. Although perturbations in the composition or function of the microbiota are linked to inflammatory and metabolic disorders (e.g. inflammatory bowel disease, irritable bowel syndrome and obesity), it is unclear at this point whether these changes are a symptom of the disease or a contributing factor. A better knowledge of the mechanisms through which changes in microbiota composition promotes disease states is needed in order to improve our understanding of the causal relationship between the gut microbiota and disease. While evidence of the preventative and therapeutic effects of probiotic strains against diarrhoeal illness and other intestinal conditions is promising, the exact mechanisms of the beneficial effects are not fully understood. Recent studies have questioned whether non-viable probiotic strains can exert health benefits on the host by influencing the immune system. As the potential health effect of these non-viable bacteria depends on whether the mechanism of the health effect is dependent on viability, future research needs to consider each probiotic strain on a case-by-case basis. The present review provides a comprehensive, updated overview of the human gut microbiota, the factors influencing its composition, and the role of probiotics as a therapeutic modality in the treatment and prevention of diseases and/or restoration of human health.

2.0 Introduction

The human intestinal microbiota plays a key role in numerous metabolic, physiological, nutritional and immunological processes (O'Hara & Shanahan, 2006), and perturbations in the composition of the microbiota influences human health (O'Toole & Claesson, 2010). Much of the early information regarding the intestinal microbiota came from studies that used culture-dependent techniques which reveal only a minority of species constituting the microbial population (Adlerberth & Wold, 2009; O'Toole & Claesson, 2010). However, the advent of culture-independent, DNA-based analyses has generated data which can be mined for information on the composition and functional properties for this hitherto-uncultured microbiota (Ehrlich, 2010; O'Toole & Claesson, 2010; Rastall, 2004).

The microbial content of the gastrointestinal tract (GIT) changes along its length, ranging from a narrow diversity and low numbers in the stomach to a wide diversity and high numbers in the large intestine (Isolauri *et al.*, 2004; Tiihonen *et al.*, 2010) (Fig. 1). The best studied region of the gut is the distal colon, and in adults, faecal-derived populations are estimated to consist of 10^{13} to 10^{14} microorganisms, composed of approximately 1,100 prevalent species, with at least 160 such species per individual. In its entirety, the microbiota is estimated to contain 150-fold more genes than the human genome (Qin *et al.*, 2010). The majority of bacteria belong either to the phylum *Firmicutes* (including *Clostridium, Enterococcus, Lactobacillus* and *Ruminococcus*) or *Bacteroidetes* (including *Bacteroides* and *Prevotella* genus) which constitute over 90% of the known phylogenetic categories found in the human intestine (Eckburg *et al.*, 2005; Hayashi *et al.*, 2002b; Hold *et al.*, 2002; Qin *et al.*, 2010; Rajilić-Stojanović *et al.*, 2009; Tap *et al.*, 2009; Wang *et al.*, 2003). Although there is huge range in inter-individual variability in microbial composition (Eckburg *et al.*, 2005; Hayashi *et al.*, 2002b; Hayashi *et al.*, 2003; Qin *et al.*, 2010), recent work has revealed that a core of >50 taxa are found in nearly half of human subjects sampled (Qin *et al.*, 2010; Tap *et al.*, 2009). It has also been suggested that the microbiota of most individuals can be categorised into three predominant variants, or 'enterotypes', dominated by three different genera: *Bacteroides*, *Prevotella* and *Ruminococcus* which are independent of age, gender, nationality and body mass index (Arumugam *et al.*, 2011). This concept was partially supported by Wu *et al.* (2011) who identified two enterotypes,

distinguished primarily by levels of *Bacteroides* and *Prevotella*, which were largely driven by diet. More recently, considerable debate has arisen about the notion of enterotypes (Jeffery *et al.*, 2012; Yong, 2012) with a number of studies (Claesson *et al.*, 2012; Huse *et al.*, 2012) failing to identify the three distinct categories described by Arumugam *et al.* (2011). Researchers are now favouring the idea of a continuum or gradient of species functionality rather than discontinuous variation with segregated types (Jeffery *et al.*, 2012).

Studies have also identified a core microbiome at the gene rather than at the organismal lineage level (Serino *et al.*, 2012; Turnbaugh *et al.*, 2009a). These studies suggest that, rather than a core group of species, individuals share a core microbiome function and individuals exhibiting particular phenotypes (e.g. obese or non-obese) may display different patterns of gut microbes but share a core of functions (Turnbaugh *et al.*, 2009a). Changes in this core set of genes may account for different states of health and disease. Future research will investigate whether the metagenome predicts a risk for developing particular human diseases to obtain new microbial diagnostic markers that may allow early diagnosis of diseases and development of potentially new therapeutic strategies.

Although much has been discovered in the last decade about the intestinal microbiota, there are biases and limitations to the current knowledge related to study design, sample collection and confounding variables, such as diet. Investigations into the impact of diet on the intestinal microbiota are challenged by the inability to carry out large-scale, carefully controlled trials in humans. There is also a need to better clarify the mechanisms through which microbial dysbiosis promotes disease states in order to improve our understanding of the causal relationship between the gut microbiota and disease. Whether a disease-prone microbial composition can be transformed to a healthier composition by probiotic/prebiotic/dietary interventions remains a fundamental unanswered question. Nonetheless the emergence and growing accessibility of the next generation sequencing technologies will greatly advance the discovery of the composition and functional capacity of microbial communities in the human gut. In this review, we discuss recent insights into the impact of age, diet, antibiotic use and disease on the intestinal microbiota. We also highlight the role of probiotics, in particular, the potential role of dead or inactivated microbes as a

therapeutic modality in the treatment and prevention of diseases and/or restoration of human health.

3.0 Factors influencing the composition of the microbiota

3.1 Age

Development of the human microbiota is a dynamic process, with different life stages exhibiting differences in terms of diversity and variation (Fig. 1) (Biagi *et al.,* 2012; O'Toole & Claesson, 2010; Tiihonen *et al.,* 2010). The human microbiota is established at birth when the intestine becomes inhabited by a population that is characterised by instability (Tiihonen *et al.,* 2010). Initially, facultative anaerobes such as *Enterobacteriaceae*, streptococci and staphylococci dominate (Marques *et al.,* 2010). In recent years, more stringent hygienic conditions during delivery combined with shorter hospital stays have reduced bacterial exposure leading to changes in the initial colonisation pattern, with skin-derived staphylococci being the first colonisers of the infant gut rather than faecal *Enterobacteriaceae* (Marques *et al.,* 2010; Morelli, 2008). For infants born vaginally, the first encounter with microorganisms occurs in the birth canal where colonisation is initiated by the maternal vaginal and intestinal microbiota as well as the environment (Lupp & Finlay, 2005). In contrast, for infants delivered by caesarean section, the environment (e.g. nursing staff and the air) is an extremely important source of colonizing bacteria, and these infants have lower intestinal bacterial counts with less diversity in the early weeks of life (Azad *et al.,* 2013; Grölund *et al.,* 1999; Morelli, 2008).

Other factors influencing the microbiota include gestational age, hospitalisation of the infant, antibiotic use and infant feeding. Breast-fed infants have a microbiota dominated by *Bifidobacterium* (Bezirtzoglou *et al.,* 2011; Penders *et al.,* 2006; Turroni *et al.,* 2012; Yatsunenko *et al.,* 2012) and *Ruminococcus* (Favier *et al.,* 2002; Morelli, 2008), with rates of colonization by *Escherichia coli, Clostridium difficile, Bacteroides fragilis*-group bacteria and lactobacilli significantly lower than those for exclusively formula fed infants (Penders *et al.,* 2006; Yoshioka *et al.,* 1983). The microbiota of formula-fed infants is more complex (Agans *et al.,* 2011; Bezirtzoglou *et al.,* 2011) and includes a variety of bacterial genera, including enterobacterial genera, *Streptococcus, Bacteroides* and *Clostridium*, *Bifidobacterium* (Favier *et al.,* 2002) and *Atopobium* (Bezirtzoglou *et*

al., 2011). It must be noted that some reports have found no differences in the microbiota between breast- and formula-fed infants and have attributed this to modern formulas more closely mimicking the composition of human breast milk (Adlerberth & Wold, 2009). The composition of the microbiota changes further with the introduction of solid food and a complex, more stable community similar to the adult microbiota becomes established after weaning (at 2-3 yrs of age) (Collins & Gibson, 1999; Favier *et al.,* 2002; Koenig *et al.,* 2011; Yatsunenko *et al.,* 2012).

Throughout adult life, the composition of the intestinal microbiota is relatively stable and is only transiently altered by external disturbances (Delgado *et al.,* 2006) as will be discussed below. However, this relative stability is reduced in old age (Tiihonen *et al.,* 2010). There is considerable variation in the reported microbiota compositions of elderly subjects which appear to be dependent on residence cohort, geographical location and detection methods used (Mueller *et al.,* 2006; Tiihonen *et al.,* 2010). The large inter-individual variation in the microbiota composition continues into old age (Claesson *et al.,* 2011) and the process of ageing coincides with decreasing microbiota diversity (Biagi *et al.,* 2010). Researchers are continually striving to elucidate the composition of the intestinal microbiota of the elderly but as yet no specific 'common core' has been identified. However, some of the fundamental changes that occur include a decrease in total number and species diversity of bifidobacteria and bacteroides as well as a reduction in amylolytic activity and the availability of total short chain fatty acids (SCFA). There is a concurrent increase in facultative anaerobes, fusobacteria, clostridia and eubacteria as well as an increase in proteolytic activity (Cusack & O'Toole, 2010; Woodmansey, 2007). A study by the ELDERMET consortium found that the microbial population of elderly Irish subjects is dominated by *Bacteroidetes*, whereas the microbiota of younger subjects is dominated by *Firmicutes* (Claesson *et al.,* 2011). However, Biagi *et al.* (2010) did not find significant differences among the *Firmicutes/Bacteroidetes* ratios of Italian centenarians, elderly and young adults. These conflicting results have been attributed to the country-related variation in the gut microbiota (Biagi *et al.,* 2012) which was highlighted several years ago (Mueller *et al.,* 2006) and presumably may be linked to diet. Furthermore, the composition of the gut microbiota of the elderly may also vary depending on residence location (Bartosch *et al.,* 2004; Claesson *et al.,* 2012), which is a proxy measure for radically different diets (see below).

3.2 Diet

Diet is a factor which undoubtedly influences the composition of the intestinal microbiota. Diet provides nutrients both for the host and the bacteria in the GIT. Most of the enzymes needed to break down the structural polysaccharides in plant material are not encoded by mammalian genomes. The intestinal microbiota produces a larger collection of degradative enzymes and exhibits a broader range of metabolic capabilities (Flint *et al.*, 2012). It is estimated that 20-60g of dietary carbohydrates reaches the colon on a daily basis (Flint *et al.*, 2012) including resistant starch, non-starch polysaccharides (NSP), plant cell wall polysaccharides and non-digestible oligosaccharides (Flint *et al.*, 2012; Fuller, 1991; Scott *et al.*, 2008). Some dietary protein (e.g. collagen and elastin) as well as various secondary plant metabolites (e.g. polyphenolic substances), can also reach the large intestine and may undergo bacterial transformations (Guarner & Malagelada, 2003; Louis *et al.*, 2007).

Alternative substrates can give rise to different products due to fermentation via different metabolic processes, while the same substrate can be metabolised by different pathways depending on their rate of supply, or the physiology and environment of the bacterial cell (Louis *et al.*, 2007). Changes in the composition of the gut microbiota in response to dietary intake occur because different bacterial species are better equipped (genetically) to utilise different substrates (Scott *et al.*, 2008). Generally, bacteria favour carbohydrates as primary energy sources if they are available (Apajalahti, 2005). Metagenomic sequencing of the intestinal microbiota identified a large group of carbohydrate active enzymes (CAZymes) (Kurokawa *et al.*, 2007). While certain species, particularly among the *Bacteroidetes*, possess large numbers of genes encoding CAZymes, which allows them to switch between different energy sources, other groups encode fewer CAZymes and are noticeably more specialised (Flint *et al.*, 2012). Dietary supplementation with prebiotics such as inulin and fructo-oligosaccharide can promote specific groups of bacteria, including bifidobacteria (Flint *et al.*, 2007; Gibson, 1999; Roberfroid, 2007). A recent study demonstrated rapid and reversible changes in the relative abundance of specific dominant bacterial groups after changes in the major type of non-digestible carbohydrate (i.e. resistant starch, NSP or reduced carbohydrate diet). There were profound inter-individual differences in the response of the microbial community to dietary change due to the inter-individual differences in the initial

microbiota composition; this suggests that dietary advice on the consumption of non-digestible carbohydrates may need to be personalised in the future (Walker *et al.,* 2010).

Saccharolytic bacterial fermentation mainly takes place in the proximal colon (due to greater fermentable carbohydrate availability) (Hamer *et al.,* 2008) and may result in the production of short-chain-fatty acids (SCFAs) (Guarner & Malagelada, 2003), the type and levels of which depend on the source and quantity of carbohydrate available, and the microbiota present (Scott *et al.,* 2008). SCFAs are energy sources for the colonic epithelium, and butyrate in particular exerts important effects on cell differentiation and gut health (Hamer *et al.,* 2008). Proteolytic fermentation generally takes place in the distal colon (where fermentable carbohydrates become depleted) (Hamer *et al.,* 2008) and results in the production of SCFAs in addition to ammonia, amines, phenols, thiols and indoles (Guarner & Malagelada, 2003).

Early studies comparing dietary patterns (e.g. 'Japanese' versus 'Western') or examining the impact of changing the proportions of food categories on the intestinal microbiota found only moderate effects involving a few genera (Drasar *et al.,* 1973; Drasar *et al.,* 1976; Finegold *et al.,* 1974). These studies relied on culture-based techniques and were therefore limited in their ability to detect changes in the fine detail of gut microbiota composition. More recent studies have employed culture-independent approaches and have further elucidated the role of diet in determining the composition of the intestinal microbiota in humans (Table 1).

In a landmark study, De Filippo *et al.* (2010) compared the faecal microbiota of European children (EU) (consuming a 'Western' diet) with that of children in the African state of Burkina Faso (BF) (consuming a plant-rich, 'rural' diet, high in fibre content). The BF children had a lower abundance of phylum *Firmicutes* and a higher abundance of phylum *Bacteroidetes* (mainly *Prevotella* and *Xylanibacter)* in their faecal microbiota compared to the EU children, who had higher levels of *Enterobacteriaceae*. *Prevotella* and *Xylanibacter,* which contain genes for cellulose and xylan hydrolysis, were associated with increased faecal SCFAs. The authors postulated that the gut microbiota co-evolved with the plant-rich diet of the BF children, allowing them to maximise energy extraction from dietary fibre, while also protecting these children from inflammation and non-infectious intestinal disease.

Similar dietary associations were found in a study linking dietary patterns of American adults with gut microbial enterotypes, dominated by *Bacteroides* or *Prevotella.* Wu *et al.* (2011) found that the *Bacteroides* enterotype was positively associated with protein and animal fat, whereas the *Prevotella* enterotype was associated with a diet high in carbohydrates and low in meat and dairy.

Vegetarianism has also been shown to alter the intestinal microbiota (Hayashi *et al.,* 2002a; Kabeerdoss *et al.,* 2011; Liszt *et al.,* 2009). The higher intakes of carbohydrate and fibre associated with this dietary practice, result in SCFA production by microbes which lowers the intestinal pH, preventing growth of potentially pathogenic bacteria such as *E. coli* and other members of *Enterobacteriaceae* (Zimmer *et al.,* 2011). Indeed, Zimmer *et al.* (2011) demonstrated that subjects consuming a vegan or vegetarian diet had lower stool pH than controls and total counts of culturable *Bacteroides* spp., *Bifidobacterium* spp., *E. coli* and *Enterobacteriaceae* spp. were significantly lower in vegan samples than in controls. A vegetarian diet has also been shown to decrease the amount and change the diversity of *Clostridium* cluster IV and *Clostridium* rRNA clusters XIVa and XVII (Hayashi *et al.,* 2002a; Liszt *et al.,* 2009).

It was recently reported that diverse dietary patterns are responsible for the gut microbiota variation seen between community-dwelling elderly subjects and subjects in long-term residential care. The diet of community-dwelling individuals was typically more diverse with low to moderate fat and high fibre intake, whereas that of subjects in long term residential care was less diverse with moderate to high fat and low to moderate fibre intake. Those in long-term care had a less diverse microbiota with a higher proportion of the phylum *Bacteroidetes,* while the microbiota of community subjects was more diverse with a higher proportion of phylum *Firmicutes.* Community-dwelling subjects had a higher abundance of bacteria from the genus *Prevotella*, supporting the association between *Prevotella* and a diet high in carbohydrates as observed in the BF children and American adults. *Coprococcus* and *Roseburia* were also more abundant in the faecal microbiota of community subjects, whereas *Parabacteroides, Eubacterium, Anaerotruncas, Lactonifactor* and *Coprobacillus* were more abundant in subjects in long-term care. For subjects in long-term care, both the faecal microbiota and the diet were associated with the duration of stay, with subjects' resident for more than a year having a diet and microbiota that was furthest separated

from community-dwelling subjects compared to recently admitted subjects. Interestingly, the major trends in the microbiota that separated the community-dwelling elderly from the elderly in long-term care were associated with changes in frailty, inflammation and other clinical markers and hence indicate a role for diet-driven microbiota alterations in health among the elderly (Claesson *et al.*, 2012).

Changes in the gut microbiota of (humanised germ-free) mice have been analysed after the mice were switched from a diet low in fat and rich in plant polysaccharides to a 'Western' diet high in fat and sugar and low in plant polysaccharides. After just a single day, mice on a 'Western' diet displayed an increased abundance of bacteria of the *Firmicutes* phylum and a decrease of those of the *Bacteroidetes* phylum (Turnbaugh *et al.*, 2009b). Hildebrandt *et al.* (2009) also found distinctive changes in the gut microbiota of mice following a switch from a standard chow to a high fat diet, which was associated with a proportional decrease in phylum *Bacteroidetes* and an increase in both *Firmicutes* and *Proteobacteria*.

Faith *et al.* (2011) developed a statistical model for predicting how a change in diet would alter the abundance of particular species of the gut microbiota. A model community of 10 genome-sequenced human intestinal bacteria (including *Bacteroides thetaiotaomicron, Bacteroides ovatus, Bacteroides caccae, Escherichia. coli, Desulfovibrio. piger, Collinsella. aerofaciens, Clostridium symbiosum, Blautia hydrogenotrophica, Eubacterium rectale* and *Marvinbryantia formatexigens*) was introduced into germ-free mice and the composition of the intestinal community as the mice consumed different proportions of protein (casein), fat (corn oil), polysaccharide (corn-starch) and sugar (sucrose) was monitored. Each mouse was fed a randomly selected diet with diet switches occurring every two weeks. Steady-state levels of community members were achieved within 4 days of a diet change. Notably, the total DNA yield per faecal pellet increased as the amount of casein in the host diet increased. In addition, changes in species abundance as a function of changes in the concentration of casein in the host diet were apparent for all ten species; the abundances of seven species were positively correlated with casein concentration, whereas the remaining three species (*E. rectale, M. formatexigens* and *D. piger*) were negatively correlated with casein concentration. Indeed, inspection of the most highly expressed genes of *E. rectale* and *M. formatexigens* suggested they focused on carbohydrate catabolism

whereas *D. piger* can use only a restricted number of substrates (e.g. lactate, H_2, succinate). In a follow-up experiment, involving diets containing various mixtures of pureed human baby foods (i.e. foods more typically consumed in human diets), changes in species abundance that were a function of diet ingredients (e.g. apple, beef, chicken, oat, pea, peach, rice and sweet potato) were found. For example, *B. ovatus* increased in absolute abundance with increased concentration of oats in the diet, whereas most of the bacterial species responded to multiple ingredients.

Although these results from animal studies are interesting, it can be difficult to apply these findings to humans due to the artificial nature of these experiments compared to natural human microbiota and food consumption patterns. Only a limited number of human clinical trials have assessed the effects of dietary pattern changes on the intestinal microbiota (De Palma *et al.*, 2009; Muegge *et al.*, 2011; Walker *et al.*, 2010; Wu *et al.*, 2011). In a controlled-feeding study with 10 individuals (*Bacteroides* enterotype), Wu *et al.* (2011) found that the microbiome composition changed detectably within 24 h of initiating a high-fat/low-fibre or low-fat/high-fibre diet, showing the rapid effect that diet can have on the intestinal microbiota. However, enterotype identity remained constant, with no stable changes to the *Prevotella* enterotype indicating that alternative enterotype states are associated with long-term diet.

In overweight men, supplementation of the diet with resistant starch increased faecal levels of *Eubacterium rectale* and *Ruminococcus bromii*, which correlates with fibre fermentation. However, the inter-individual variation in microbiota response to resistant starch indicates that dietary interventions may need to be personalised (Walker *et al.*, 2010).

In another study, a gluten free diet (GFD) intervention which featured a reduction in overall polysaccharide intake, led to reductions in gut bacteria populations such as *Bifidobacterium, Clostridium lituseburense* and *Faecalibacterium prausnitzii* and proportional increases in *E. coli* and total *Enterobacteriaceae* in healthy volunteers (De Palma *et al.*, 2009; Sanz, 2010).

Based on the available data, differences in the composition of GI microbiota are demonstrable between groups of people living on different diets. These diet-associated changes in the composition can lead to changes in metabolic activity of the intestinal

microbiota which may in turn provoke changes in inflammatory and immune responses. Although attempts to change the composition of the intestinal microbiota by varying the diet have been successful in mice, there is a relative paucity of human dietary intervention studies and those available are small in sample size and took place over a short period of time. Moreover, mechanisms that link dietary changes to microbiota alterations remain poorly defined and need to be further investigated. Large, well-controlled trials are also required to determine the impact of altering long-term dietary patterns on the human intestinal microbiota and to elucidate the implications of the key population changes for health and disease.

3.3 Antibiotics

Antibiotic treatment has been shown (Claesson *et al.,* 2011; De La Cochetiere *et al.,* 2008; Woodmansey *et al.,* 2004; Young & Schmidt, 2004) to dramatically disturb the composition of the faecal microbiota in humans. Palmer *et al.* (2007) reported changes in the density or composition of the intestinal microbiota in infants following antibiotic treatment. Striking changes were found in some cases, even to a point where the faecal microbiota was undetectable. As there is considerable inter-individual variability in the composition of the microbiota among humans (Claesson *et al.,* 2011), it has been suggested that the impact of antibiotics is best assessed on an individual basis (Jernberg *et al.,* 2010). In general, antibiotic treatment leads to a decrease in the diversity of the microbiota (Jernberg *et al.,* 2007). Nonetheless, the community is quite resilient and can resemble the pre-treatment state in a matter of days or weeks (Claesson *et al.,* 2011; De La Cochetiere *et al.,* 2005; Dethlefsen *et al.,* 2008) However, a number of other studies have shown that the microbiota alterations following antibiotic administration can often persist for a long time following withdrawal of treatment, with some members of the microbial community failing to return to pre-treatment levels and these may even be lost from the community indefinitely (Croswell *et al.,* 2009; Dethlefsen *et al.,* 2008; Dethlefsen and Relman, 2011; Jakobsson *et al.,* 2010; Jernberg *et al.,* 2010). Microbiota disruption by antibiotics can also affect the metabolic activity of the bacterial community in the gut. Antibiotic treatment in mice was shown to drastically alter the intestinal metabolome by affecting host metabolic pathways such as sugar, nucleotide and fatty acid metabolism as well as bile acid, eicosanoid, and steroid hormone synthesis coding capacity (Antunes *et al.,* 2011).

The effect of antibiotics on the intestinal microbiota in infancy is of particular concern. Recent reports demonstrated that short-term parenteral antibiotic treatment of neonates caused significant alternations in the gut microbiota including a disturbance of the expected colonisation pattern of bifidobacteria (Fouhy *et al.*, 2012; Hussey *et al.*, 2011). Colonisation of the intestine early in life has an important role in directing immune system development, and antibiotic use may increase the risk of atopy and allergic asthma by reducing the protective effect of microbial exposure (Foliaki *et al.*, 2009; Russell *et al.*, 2012). In a large, multi-centre study, Foliaki *et al.* (2009) reported an association between antibiotic use in the first year of life and symptoms of asthma, rhinoconjunctivitis and eczema in children 6 and 7 years old.

The impact of antibiotic use on the intestinal bacteria of the elderly is also of interest since, compared with younger adults, cohorts of elderly populations are typically administered a complex array of medications, including antibiotics. Antibiotic treatment in hospitalised elderly subjects was shown to increase the intestinal abundance of proteolytic bacteria (Woodmansey *et al.*, 2004), to reduce overall bacterial numbers, and in some subjects, to completely eliminate certain bacterial communities (Bartosch *et al.*, 2004). A more recent study found that antibiotic treatment led to a decrease in the taxonomic richness, diversity, and evenness of the intestinal community in elderly subjects, although the magnitude of the changes and the taxa affected were different between subjects. Moreover, the overall community structure was restored within 4 weeks post-treatment (Claesson *et al.*, 2011).

One of the best known complications arising following antibiotic therapy is antibiotic-associated diarrhoea (AAD) (Sekirov *et al.*, 2010). A number of mechanisms underlie the development of AAD. Antibiotic therapy can disturb the natural microbiota in the GIT, which may result in pathological overgrowth of *Cl. difficile* and it may also disturb the metabolism of carbohydrates, giving rise to maladsorption of osmotically active particles (Vanderhoof *et al.*, 1999; Young & Schmidt, 2004). Young & Schmidt (2004) found that, in a patient who developed AAD, antibiotic administration was associated with distinct changes in the diversity of the gut microbiota, including a decrease in the prevalence of butyrate-producing bacteria. Following discontinuation of the antibiotic, resolution of diarrhoea was accompanied by a reversal of these changes. This provided

evidence linking changes in the community structure of the gastrointestinal bacteria with the development of AAD.

The impact of antibiotic use in the short and long-term needs to be further investigated. Longitudinal type studies rather than cross sectional studies will allow more direct testing of questions regarding the influence of antibiotic use on the development of allergy and gastrointestinal disease, particularly in early life.

3.4 Disease

3.4.1 Inflammatory Bowel Disease

Inflammatory Bowel Diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic intestinal disorders whose aetiology is unclear. However, an abnormal immune response against luminal antigens, such as dietary factors and/or bacteria may be involved (Isaacs & Herfarth, 2008; Ojetti *et al.,* 2009). CD can affect any part of the GIT, although the lower ileum and colon are most commonly involved (Reiff & Kelly, 2010). It is characterised by discontinuous inflammation of the epithelial lining and deep ulcers. UC affects only the colon and the rectum and is characterised by continuous mucosal inflammation and superficial ulcers (Gerritsen *et al.,* 2011). Clinical symptoms of IBD include abdominal pain, diarrhoea, rectal bleeding, malaise and weight loss (Reiff & Kelly, 2010).

Numerous studies have compared the intestinal microbiota composition of IBD patients and healthy individuals and it appears that the dominant microbiota differs between the two groups (reviewed in: Dicksved & Willing, 2011; Gerritsen *et al.*, 2011; Ojetti *et al.*, 2009; Shanahan, 2010a). Similarly, the dominant microbiota in patients with UC differs from those with CD (Gerritsen *et al.,* 2011; Ojetti *et al.,* 2009). Some changes in the microbiota composition are however shared between UC and CD patients (Dicksved & Willing, 2011).

Although the phylum level changes observed in IBD patients have not always been consistent, in general an overall decrease in microbial diversity and stability of the intestinal microbiota has been observed in IBD patients (Dicksved & Willing, 2011; Gerritsen *et al.,* 2011). A decrease in the abundance of specific members of the *Firmicutes* has been reported, which in some cases coincided with an increase in

Bacteroidetes abundance and that of facultative anaerobes such as *Enterobacteriaceae* (Gerritsen *et al.,* 2011). Moreover, increased numbers of *E. coli,* some of which may be pathogenic have been observed in IBD patients (Dicksved & Willing, 2011). Increased detection of *Cl. difficile* in relapse and remission of both forms of IBD has been observed (Shanahan, 2010a). Other reports described alterations in abundance of *Bacteroidetes* spp., proteobacteria, bifidobacteria and lactobacilli but results have been inconsistent (Dicksved & Willing, 2011).

As regards CD, a number of consistent observations have been reported (Shanahan, 2010a). These include increased mucosal bacterial counts, detection of increased levels of adherent-invasive *E. coli* (AIEC), and increased levels of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Furthermore, a reduced number of bacteria in the *Clostridium leptum* group, including *Faecalibacterium prausnitzii,* have been observed in CD patients (Sokol *et al.,* 2008). In fact, *F. prausnitzii* was even proposed as a potential probiotic for counterbalancing dysbiosis in CD (Sokol *et al.,* 2008). As for UC, a reduced presence of the *Clostridium coccoides* group has been described but no specific members of this group have been correlated with the disease as yet (Dicksved & Willing, 2011).

Although marked alterations occur in the gut microbiota composition of IBD patients, it is unclear whether these shifts cause the disease or whether they arise due to the changes in the gut environment that result from the disease. Indeed most of the studies carried out to date have made associations between the microbiota and IBD only after the IBD phenotype has emerged which doesn't allow one to answer the important question of what came first – IBD or a change in the microbiome. More long-term longitudinal studies are needed to examine the disease progression and to typify the taxonomic and functional composition changes of the microbiome that lead to, or may even define IBD.

3.4.2 Irritable Bowel Syndrome

Irritable Bowel Syndrome (IBS) is a common, debilitating gastrointestinal disorder characterised by abdominal pain, bloating and disturbances in bowel function (Jeffery *et al.,* 2011; Madden, 2004; Malinen *et al.,* 2005). IBS can present as diarrhoea-

predominant IBS (D-IBS), constipation-predominant IBS, or mixed-bowel-habit IBS (Carroll *et al.*, 2011).

IBS can be difficult to diagnose due to a lack of a biological or pathogenic marker (Madden, 2004; Malinen *et al.*, 2005). Although the pathophysiology of IBS is still not well understood, several factors are thought to play a role. These include: mal-fermentation of food ingredients; an altered microbiota; intestinal motor and sensory dysfunction; immune mechanisms; psychological factors and brain-gut axis dysregulation (Andresen & Baumgart, 2006; Madden, 2004; Quigley, 2007). Considerable evidence suggests that factors that disturb the gut microbiota, such as gastroenteritis, may contribute to the development of IBS (Thabane *et al.*, 2007).

The differences in the intestinal microbiota between IBS patients and healthy controls (HCs) have mostly been studied using faecal material. Mättö *et al.* (2005), using culture based techniques, observed slightly higher numbers of culturable coliforms and an increased aerobe:anaerobe ratio in IBS subjects relative to healthy controls. Moreover, PCR-DGGE revealed more temporal instability in the predominant bacterial population of IBS subjects than in controls and IBS subjects had more *Clostridium* spp. and less *Eubacterium* spp. amplicons. However, the researchers did not control for antibiotic use, which may have contributed to the apparent temporal instability observed (Mättö *et al.*, 2005). In a subsequent study, which targeted the clostridial groups in IBS, it was reported that a similar instability existed (Maukonen *et al.*, 2006). In addition, a study employing DGGE techniques found that there was significantly more variation in the gut microbiota of healthy volunteers than that of IBS patients (Codling *et al.*, 2010).

Jeffery *et al.* (2011) described detailed analysis of the faecal microbiota in a cohort of well characterised IBS patients and control subjects and found no uniform change in the microbiota in IBS. However, analysis of the microbial populations revealed distinct clusters, one of which showed normal-like microbiota composition compared with healthy controls. The other IBS samples were characterised by an increase of *Firmicutes*-related taxa and a decrease of *Bacteroidetes*-related taxa. In addition, analysis of the IBS microbiota and separate analyses of the two subgroups showed microbial associations with colonic transit time, satiety, bloating, rectal pain threshold and depression (Jeffery *et al.*, 2011). Significantly, the IBS subjects with a microbiota similar to that of the matched healthy controls displayed higher anxiety and depression

scores, suggesting a non-intestinal or at least a non-microbiota aetiology for IBS in this sub-group.

A more recent study showed intestinal dysbiosis in diarrhoea-predominant IBS patients compared to healthy controls. A significant increase in the abundance of unclassified *Enterobacteriaceae* and significant reductions in members of the *Faecalibacterium* genus were found in IBS patients compared to controls. Furthermore, *Enterococcus, Fusobacterium, Pediococcus,* unclassified *Lactobacillaceae* and *Veilonella* species were found in IBS patients but were below detection limits in the healthy controls (Carroll *et al.,* 2012).

The studies described above demonstrate that the intestinal microbiota of patients with IBS can differ from that of healthy individuals. Nonetheless, it is not yet possible to be certain (as in IBD above) whether the alterations in the intestinal microflora seen in IBS patients are the cause of IBS, or are simply a result of the disrupted gut motility or other physiological features of IBS. More studies are needed to clarify whether the microbiota have a causal role in the initiation and/or progression of IBS.

3.4.3 Obesity

Some of the earliest evidence showing the role of the gut microbiota in the regulation of fat storage was demonstrated in animal models. A pioneering study by Bäckhed *et al.* (2004) found that germ-free (GF) mice were leaner than their conventional counterparts and colonisation with an intestinal microbiota resulted in a significant increase in body fat content despite lower food consumption in the colonised animals. A subsequent study found that GF mice were protected against obesity following consumption of a western style, high fat, sugar rich diet (Bäckhed *et al.,* 2007). In addition, colonisation of GF mice with an 'obese microbiota' (i.e. from an obese animal) led to greater increases in total body fat compared to GF mice colonised with a 'lean microbiota' (Turnbaugh *et al.,* 2006), indicating that the obese microbiota had an increased capacity to harvest energy from the diet.

It has been suggested that inflammation (Cani *et al.,* 2007) and alterations in host gene expression (Bäckhed *et al.,* 2007) are other mechanisms by which gut microbiota may influence the host. Obesity and its related metabolic disorder, type 2 diabetes, are generally associated with chronic low-grade inflammation (Wellen & Hotamisligil,

2005). The highly proinflammatory component, lipopolysaccharide (LPS) is a possible initiator of metabolic impairment (Cani *et al.,* 2007). Plasma LPS levels increase with higher fat intake in both mice (Cani *et al.,* 2007) and humans (Erridge *et al.,* 2007) and the direct infusion of LPS mimics the physiological effects of the high fat diet in mice (Cani *et al.,* 2007). It has been hypothesised that LPS is taken up with dietary fats in chylomicrons (Ghoshal *et al.,* 2009) or that LPS reaches the circulation because the gut is more permeable in obese mice due to disruption of tight junction proteins (Brun *et al.,* 2007; Cani *et al.,* 2009). A review on this topic has been described recently (Sommer & Bäckhed, 2013).

Studies have also linked alterations in the intestinal microbiota composition to obesity (Duncan *et al.,* 2008; Ley *et al.,* 2005; Ley *et al.,* 2006; Zhang *et al.,* 2009). An increased ratio of *Firmicutes* to *Bacteroidetes* has been observed in genetically obese mice (ob/ob) (Ley *et al.,* 2005) as well as obese humans (Ley *et al.,* 2006; Turnbaugh *et al.,* 2009a). However, a number of other studies have failed to confirm these findings and have shown variable patterns in the phylum level changes measured in the microbiota composition of obese humans (Duncan *et al.,* 2007; Duncan *et al.,* 2008; Schwiertz *et al.,* 2010).

Although it is clear from the studies described above that the gut microbiota are likely to play some role in obesity and metabolic disease, it is difficult to draw definite conclusions on the importance of particular bacterial groups. Further well-designed, large clinical studies will be required to identify microbiota-related biomarkers of risk for obesity and metabolic dysregulation.

4.0 Manipulation of intestinal microbiota

4.1 Probiotics

The term ‘probiotic’, a word derived from the Greek and meaning ‘for life’ (Quigley, 2007), has been defined as ‘*live microorganisms which when administered in adequate amount confer a health benefit on the host*’ (FAO/WHO, 2002). Some probiotic products contain a single strain while others contain a mixture of several species of bacteria or fungi. The most studied and commonly used organisms in probiotic preparations are lactobacilli and bifidobacteria.

One of the original concepts associated with probiotics was that their consumption would alter the composition of the intestinal microbiota from a possibly harmful one towards a microbiota that would benefit the host (i.e. replace 'bad' bacteria with 'good' bacteria) (Metchnikoff, 1908). This was a rather simplistic theory, and it was not based on a full understanding of the complexity of the intestinal microbiota anyway. It has since been suggested (Ouwehand *et al.,* 2002) that too much emphasis should not be placed on the potential change in microbiota composition but rather the inherent health benefits conferred by the probiotics themselves. Indeed for some probiotic effects (e.g. immune modulation) it may not be necessary to achieve a measurable modification of the intestinal microbiota composition. In recent times two main motives have emerged for the use of probiotics. The first one is the use of probiotics by healthy subjects in order to maintain a healthy state and decrease the risk of illness. The second is the use of probiotics as a treatment/therapeutic modality targeted at particular diseases.

There is a variety of proposed health effects (both direct and indirect) for probiotics which have been extensively reviewed (Goldin, 2011; Parvez *et al.,* 2006; Vanderhoof & Young, 2008). However, the subtleties of the positive effects of probiotics can only be fully appreciated following meta-analysis. Some of the most robust clinical data are confined to the preventative and therapeutic effects of probiotic strains against diarrhoeal illnesses (Allen *et al.,* 2011; Bernaola Aponte *et al.,* 2010; Hempel *et al.,* 2012; Videlock & Cremonini, 2012). A number of beneficial effects of probiotics dealing with intestinal health have been evaluated in Cochrane reviews (AlFaleh *et al.,* 2010; Allen *et al.,* 2011; Bernaola Aponte *et al.,* 2010; Johnston *et al.,* 2007). These and other meta-analyses have demonstrated the efficacy of probiotics in the prevention and treatment of antibiotic-associated diarrhoea (Hempel *et al.,* 2012; Johnston *et al.,* 2007; Videlock & Cremonini, 2012), acute infectious diarrhoea (Allen *et al.,* 2011) and persistent diarrhoea in children (Bernaola Aponte *et al.,* 2010). Evidence is also accumulating from well-conducted clinical studies on the efficacy of probiotics in preventing and reducing the severity of necrotizing enterocolitis (NEC) in premature infants and those with very low birth weight (AlFaleh *et al.,* 2010; Barclay *et al.,* 2007; Wang *et al.,* 2012). Probiotics have also yielded promising improvements in the prevention and treatment of Inflammatory Bowel Diseases (IBD; Ulcerative Colitis and Crohn's Disease) (Isaacs & Herfarth, 2008) and Irritable Bowel Syndrome (IBS) (Quigley, 2007). However, it must be noted that these meta-analyses have their own

limitations. The clinical and methodological heterogeneity between studies as well as the differences in probiotic type, delivery method (yoghurt *vs.* capsule) and dosage make comparisons difficult. Indeed no two different probiotics are likely to be functionally the exact same and therefore performing meta-analyses based on studies involving different strains, species and even genera is inherently questionable. Different strains may have vastly different effects and hence no ideal probiotic strain for any of the above conditions has been identified, despite continuing advances in this area.

Although there is no single mechanism of action for probiotics, there are a number of common mechanisms by which probiotics might influence the intestinal microbiota (Fig. 2) (O'Toole & Cooney, 2008). It is likely however, that the mechanism of action of probiotics is multi-factorial and strain specific (Tuohy *et al.,* 2003).

While there are extensive scientific and clinical portfolios associated with (specific strains of) probiotics, the European Food Safety Authority (EFSA) has yet to approve health claims for a single probiotic (there have been 120 negative opinions on probiotic claims through February 2011) (Sanders *et al.,* 2011). Indeed, the regulators are applying almost pharmaceutical standards to the use of probiotics. As already mentioned, there are two main uses for probiotics: 1) probiotics as a 'food for health' product or 2) probiotics as a therapeutic modality for illness. The first example is clearly one where the food industry is focussing its resources – that is, keeping healthy people healthy. One may ask if it is appropriate for the regulators to apply pharmaceutical industry standards of proof for probiotics which are largely intended to be given to healthy people? Moreover, unlike pharmaceutical drugs with a single active entity, probiotics encompass hundreds of different strains and hundreds of different surface molecules and possibly metabolites which may be responsible for the 'probiotic' effect. Certainly this is a regulatory challenge that is yet to be resolved. As a scientist interested in exploring probiotic function, one has to be in favour of stringent regulation and the challenge is now firmly with commercial probiotic purveyors to generate high quality scientific data that will allow them to make health claims. However, one can also conclude that trying to apply the same concepts and standards as employed for pharmaceuticals may not be appropriate for probiotics. The scientists, companies and regulators need to address these issues. Otherwise, credibility with the consumer,

interest by the food industry and ultimately scientific research will be seriously damaged.

There has been recent interest in faecal transplantation as an alternative approach for the manipulation of the intestinal microbiota. Indeed, evidence for its use as a treatment for gastrointestinal illness (including pseudomembranous colitis, *Cl. difficile*-associated diarrhoea; antibiotic associated diarrhoea, IBS and IBD) is rapidly accumulating and has been reviewed recently (Anderson *et al.,* 2012; Guo *et al.,* 2012; Landy *et al.,* 2011). Faecal transplantation as a treatment modality remains a controversial and the available evidence for its efficacy is limited. Nonetheless this therapy holds great promise and further studies are necessary to explore this potential.

4.1.1 Probiotics: dead or alive?

It has been proposed that the minimum therapeutic dose for probiotics is 10^8-10^9 viable cells per day (Kailasapathy & Chin, 2000). However, live cells in probiotic products will inevitably lose viability and the actual products will contain varying proportions of populations of viable to non-viable/dead cells (Shah *et al.,* 1995). There may be further losses of viability of the organisms on passage through the relatively hostile environment of the stomach and small intestine (Adams, 2010). Concerns have also been raised that the administration of live microorganisms may not be appropriate for some population groups (e.g. premature infants and immunocompromised individuals) as they may translocate to the locally draining tissues, thereby causing bacteraemia and sepsis (Kataria *et al.,* 2009; Lopez *et al.,* 2008).

An area of related on-going debate is therefore whether or not non-viable forms of beneficial bacterial strains have a role in conferring benefits to the host. Indeed, a considerable amount of published scientific evidence indicates that inactivated microbes may positively affect health by influencing the host immune system (reviewed in: Adams, 2010; Kataria *et al.,* 2009; Taverniti and Guglielmetti, 2011). The ability of bacterial cells to potentially interact with the host, independent of viability, is based on the capacity of human cells to recognise specific bacterial components or products, leading to responses that commonly involve the mucosa-associated lymphoid tissue, and therefore, the immune system (Adams, 2010). Some studies have proposed that the immunomoddulatory effects exerted by non-viable probiotics may be due to their

immunostimulatory DNA, cell wall components, peptidoglycan, intra- and extra-cellular polysaccharide products and cell-free extracts (Dalpke *et al.*, 2002; Hosono *et al.*, 1997; Lammers *et al.*, 2003; Rachmilewitz *et al.*, 2004).

A number of studies have evaluated the immunomodulatory effect of probiotic *L. rhamnosus* GG (LGG), in both live and dead (inactivated) form. Heat-killed or ultra-violet inactivated LGG may reduce inflammation by decreasing experimentally induced IL-8 production by epithelial Caco-2 cells (Lopez *et al.*, 2008; Zhang *et al.*, 2005). In the absence of induction, high doses of live LGG actually induce production of IL-8, while the heat-killed agent causes only a slight increase in IL-8 meaning it had a lower potential to cause inflammation itself (Zhang *et al.*, 2005), thus indicating the heat-killed agent may be a safer alternative. A similar response was demonstrated in an animal model, in which both live and heat-killed LGG reduced LPS-induced pro-inflammatory mediators and up-regulated anti-inflammatory mediators in intestinal tissue in rats (Li *et al.*, 2009). Similarly, heat-killed *Lactobacillus* strains were found to induce secretion of TNF-α from mouse splenic mononuclear cells, to various degrees (Matsuguchi *et al.*, 2003). Furthermore, the purified surface glycolipid, lipoteichoic acid (LTA), which is a major component of the cell wall of lactobacilli, activated macrophages through TLR2, in a strain specific manner. It has even been suggested that the immense structural diversity in the LTAs derived from different bacteria may induce a variety of immunoregulatory properties (Ciszek-Lenda *et al.*, 2011).

The immunoregulatory potential of exopolysaccharide (EPS) has also been investigated (Arena *et al.*, 2006; Ciszek-Lenda *et al.*, 2011; Hidalgo-Cantabrana *et al.*, 2012; López *et al.*, 2012; Wu *et al.*, 2010). It has been shown that *in vitro* levels of pro-inflammatory cytokines are highly elevated upon exposure of mouse splenocytes to cells of EPS-deficient *Bifidobacterium breve* co-cultures, whereas exposure to an EPS-producing strain markedly reduced these cytokine levels. Moreover, treatment of mice with EPS^+ *B. breve* elicited reduced levels of pro-inflammatory immune cells compared with EPS^- strains (Fanning *et al.*, 2012). A recent study described the stimulatory effects of a *Lactobacillus*-derived EPS on the release of inflammatory mediators by mouse peritoneal macrophages *in vitro* (Ciszek-Lenda *et al.*, 2011). EPS effectively induced the production of mediators and cytokines by macrophages, especially TNF-α, IL-6 and IL-12. Interestingly, EPS induced higher levels of TNF-α and IL-6 than IL-10,

suggesting a net pro-inflammatory potential. However, its stimulatory effect was significantly lower than LPS or whole, killed bacterial cells. Moreover, whole cells were stronger inducers of anti-inflammatory IL-10 than EPS alone, suggesting that intact bacteria and EPS may have an opposing effect on macrophage polarisation (Ciszek-Lenda *et al.,* 2011). Similarly, Wu *et al.* (2010) investigated the effect of heat-killed *B. longum* and its isolated EPS fraction on the activities of a murine macrophage cell line, including IL-10 and TNF-α induction. EPS exposure stimulated growth and induced IL-10 secretion in the macrophage cells as well as inducing lower levels of TNF-α secretion. LPS on the other hand induced high levels of TNF-α secretion and EPS pre-treatment prevented LPS-induced release of TNF-α. As EPS and LPS are both surface macromolecules with oligosaccharide moieties, the authors concluded that EPS may act as an LPS blocker. Although EPS may play a role in immune regulation, information about the molecular mechanisms by which EPS interacts with the immune system is scarce (Hidalgo-Cantabrana *et al.,* 2012). It has also proved difficult to define the common biological properties of EPS because of its enormous structural diversity (Ciszek-Lenda *et al.,* 2011).

Some studies have found that bacterial DNA may be partly responsible for the immunomodulatory effect of probiotics. Administration of non-viable, irradiated probiotics (VSL#3) but not heat-killed probiotics was shown to effectively ameliorate experimental colitis in mice mediated by a TLR-9-probiotic DNA interaction (Rachmilewitz *et al.,* 2004). Similarly, *Bifidobacterium* genomic DNA induced secretion of the anti-inflammatory IL-10 by human peripheral blood mononuclear cells (Lammers *et al.,* 2003).

Immune-active components of probiotic bacteria may not be limited to cell wall structures and DNA. There have been reports concerning a soluble immunomodulator from bifidobacteria. The immunomodulating activity of *Bifidobacterium adolescentis* increases after disruption of the cells by sonication and the immunopotentiating activity appeared in the soluble fraction following centrifugation, indicating the existence of an intracellular soluble immunomodulator (Hosono *et al.,* 1997). *Bifidobacterium pseudocatenulatum* components also have immunomodulatory effects which appear to be dependent on the method of preparation (Hiramatsu *et al.,* 2007). Compared to heat-treated and untreated cells, sonicated *Bifidobacterium* was the most potent inducer of

innate immune responses in Peyer's patch (PP) cells *in vitro* and *in vivo* (following a single-shot oral administration to mice). However, heat-treated *Bifidobacterium* showed the greatest immunomodulatory activity following repeated oral administration (for 7 consecutive days) to mice. The researchers concluded that the immunomodulatory effect of *Bifidobacterium* is dependent upon the bacterial conformation and condition (Hiramatsu *et al.,* 2007).

Although there is substantial evidence from *in vitro* and animal studies that inactivated probiotics can act as biological response modifiers, there is a relative paucity of information on the effect of dead probiotics *in vivo* in human clinical trials. However, a number of basic human consumption studies have been carried out with Lacteol Fort (*Lb. acidophilus* LB which is heat-killed and freeze dried in the presence of its fermented culture medium) (Halpern *et al.,* 1996; Xiao *et al.,* 2002). Lacteol Fort was shown to improve clinical symptoms (by decreasing bowel movement, abdominal pain and distension and by improving stool consistency and the feeling of incomplete evacuation) of chronic diarrhoea (Xiao *et al.,* 2002), possibly through a mechanism involving competitive exclusion (Chauvière *et al.,* 1992).

To conclude, while a number of studies have proposed that the viability of probiotics is not essential to exert an immunomodulatory effect, this is not a uniform feature of all probiotics tested to date, as different *in vitro* studies have also reported that a viable probiotic is essential to exert an immunomodulatory effect (Arribas *et al.,* 2011; Ma *et al.,* 2004). In addition, the method of preparation may play a significant role as the immunodulatory effect may be dependent on the bacterial conformation and condition, as has been described above (Hiramatsu *et al.,* 2007; Rachmilewitz *et al.,* 2004). Further study is essential to elucidate whether inactivated probiotic bacteria or their products are able to provide similar beneficial effects as live bacteria *in vivo*. Work on the specific mechanisms is also required to explain what is actually being triggered by the dead agents and whether this is similar to the mechanisms of live agents (Li *et al.,* 2009). Biological products based on dead cells might be relatively easy to produce, commercialise, and standardise and would have the added benefit of having a much longer shelf life. In addition, use of dead probiotics may be, in some circumstances, safer than using live probiotics. Resolution of this debate might also require participation of relevant stakeholders in re-examining the definition of a probiotic,

which is currently restricted to live cells (FAO/WHO, 2002). It must be noted that while probiotic viability may not be necessary to exert an immunomodulatory effect, a number of mechanisms mediating probiotic health benefits do require viability, such as metabolism of non-digestible polysaccharides and production of metabolites (e.g. SCFAs). Hence, one should be cautious in applying the term 'probiotic' to these dead cell preparations.

5.0 Conclusion

The intestinal microbiota is undoubtedly an important factor in determining health status of the host and has been implicated in both gastrointestinal and extra-intestinal disorders. The studies described in this review raise the question of whether it is now possible to deduce the composition of a 'healthy' normal microbiota. Indeed, limited accessibility of the different parts of the GIT (including the colonic mucosa) as well as the individual-specific, complex composition of the GIT microbiota makes our understanding of this community somewhat incomplete (Zoetendal *et al.*, 2008). It is also essential to bear in mind the influence of different dietary patterns on the activity and composition on the microbiota and the potential implications for host health. Although several large international studies are striving to improve our knowledge of the composition of the gut biota, an overwhelming majority of microbes that compose these microbial communities are not yet characterised to any great level of detail (Ehrlich, 2010). Comparing data from different studies is difficult as sample processing and analysis methods vary between research groups. Moreover, our apparent inability to culture all members of the microbiota makes it currently impossible to create hypotheses regarding the role of these uncultured microbes in health and disease (Zoetendal *et al.*, 2008). As we are still unable to completely define the microbiota of a healthy intestinal tract, it is similarly difficult to define the microbiota associated with an intestinal disorder. However, major advances in human metagenomics have now provided a catalogue of 3.3 million non-redundant genes (Ehrlich, 2010; Qin *et al.*, 2010). This catalogue will enable the development of the gene profiling approaches which aim to detect associations of bacterial genes and phenotypes. These developments should lead to rapid advances in diagnostic and prognostic tools as well as paving the way to rational approaches to the manipulation of an individual's intestinal microbiota in order to promote health.

The improvement or maintenance of health through the use of probiotics has been the focus of extensive research. Indeed, the probiotic market has expanded rapidly in recent years and a large variety of probiotic products are available. However, the efficacy of probiotics is strain and dose dependent and the clinical and methodological differences (strain, dose, formulation) between studies make comparisons difficult. Hence, there is presently strong evidence to support their use in only a few conditions. It is also acknowledged that in most cases the exact mechanisms of the beneficial effects are not fully understood. Although many studies have provided several possible modes of action, it has not been possible to identify definite cause-effect relationships. Stringent requirements imposed by regulatory authorities such as EFSA require more solid scientific evidence to support any health claims associated with probiotic products. Future research requires well designed, large, randomised double-blind placebo controlled clinical trials along with more mechanistic studies in cell and animal models in order to strengthen our evidence base. Indeed the development of novel *in vitro* models of the human intestinal epithelium (e.g. bioreactors and organoids) will increase our understanding of the molecular mechanisms of host-microbe interaction and is paving the way for future ventures aimed at bioengineering human intestine (Bermudez-Brito *et al.,* 2013). Further investigation into the health benefits of ingesting dead organisms *in vivo* is also required. Indeed the potential health effect of these non-viable bacteria depends on whether the mechanism of the probiotic health effect is dependent on viability and hence each probiotic strain should be assessed on a case-by-case basis. Furthermore, as the term ‘probiotic’ fails to account for the use of dead organisms, it has been suggested that the term ‘pharmabiotic’ would be more inclusive (Shanahan, 2010b).

Table 1. Associations of the human intestinal microbiota with habitual dietary patterns or interventions

Authors	Methods	Study design	Subjects	Diets/nutrients	Microbiota response
Claesson *et al.* (2012)	16s rDNA sequencing	Cross-sectional	178 elderly subjects (64-102 yrs) - community, day-hospital, rehabilitation and long stay subjects	'Community' diet – diverse with low-moderate fat/high fibre	↑diversity ↑ *Firmicutes* ↑ *Coprococcus, Roseburia*
				'Long-Stay' diet – reduced diversity with moderate-high fat/low-moderate fibre	↓ diversity ↑ *Bacteroidetes* ↑*Parabacteroides, Eubacterium, Anaerotruncas, Lactonifactor* and *Coprobacillus*
De Filippo *et al.* (2010)	16s rDNA sequencing & biochemical analysis	Cross-sectional	29 children (1-6 yrs) - African children from Burkina Faso (n=14) and European children from Florence, Italy (n=15)	'Western' diet – high fat/protein/sugar and low fibre	↑*Firmicutes* ↑ *Enterobacteriaceae*
				'Rural' diet – low fat/protein and high fibre	↑ *Bacteroidetes* exclusively present:*Prevotella, Xylanibacter, Butyrivibrio* and *Treponema* ↑ SCFAs
De Palma *et al.* (2009)	FISH & qPCR	Feeding (1 mo)	10 healthy subjects (mean age 30.3 yrs)	Gluten-free diet (reduced polysaccharide)	↓ *Bifidobacterium, Lactobacillus, Clostridium lituseburense* and *Faecalibacterium prausnitzii* ↑ *Enterobacteriaceae* and *E. coli*
Kabeerdoss *et al.* (2011)	qPCR	Cross sectional	56 healthy female subjects (18-27 yrs) 32 vegetarian, 24 omnivore	Vegetarian diet	↓*Clostridium* cluster XIVa ↓ *Roseburia - Eubacterium rectale* ↓ butyrl-CoA CoA-transferase gene
Liszt *et al.* (2009)	qPCR & PCR-DGGE	Cross sectional	29 healthy subjects (19-34 yrs) - 15 vegetarians and 14 omnivores	Vegetarian diet	↑ bacterial DNA tendency for ↓ *Clostridium* cluster IV and ↑ *Bacteroides* (but not significant)
Muegge *et al.* (2011)	16s rDNA sequencing & shotgun metagenomics	Cross-sectional	18 lean subjects (mean age 59.6 yrs) - members of a Calorie Restriction Society	proteins	associated with KEGG orthology groups
				insoluble dietary fibre	associated with bacterial OTU content
Walker *et al.* (2010)	16s rDNA sequencing & qPCR	Randomised cross over (3 wk intervention)	14 overweight male subjects (27-73 yrs)	Diet high in resistant starch (type III)	↔ phylum level ↑*Ruminococci bromii* and *E. rectale* ↑ *Ruminococcaceae* ↑ *Oscillibacter valericigenes* ↑ *Firmicutes* bacteria related to *Roseburia* and *E. rectale*
				Reduced carbohydrate diet (weight loss diet)	↔ phylum level ↓*Collinsella aerofaciens* ↑ *Oscillibacter valericigenes* ↓ *Firmicutes* bacteria related to *Roseburia* and *E. rectale*

Wu *et al.* (2011)	16s rDNA sequencing & shotgun metagenomics	Cross-sectional	98 healthy subjects (18-40 yrs)	Fat	↑ *Bacteroidetes, Actinobacteria* ↓*Firmicutes, Proteobacteria*
				Fibre	↓*Bacteroidetes, Actinobacteria* ↑ *Firmicutes, Proteobacteria*
				Animal fat & protein	positively associated with *Bacteroides* enterotype
				Carbohydrates	positively associated with *Prevotella* enterotype
Wu *et al.* (2011)	16s rDNA sequencing & shotgun metagenomics	Controlled feeding (10 d intervention)	10 subjects in *Bacteroides* enterotype (high fat/protein)	low fat/high fibre diet or high fat/low fibre	changes in microbiome composition detectable within 24 h of initiating diet; no stable switch in enterotype after 10 days
Zimmer *et al.* (2011)	culture-based methods	Cross sectional	295 healthy subjects - 144 vegetarians, 105 vegans, 46 controls	Vegetarian diet	↓ stool pH
				Vegan diet	↓ stool pH ↓ *Bacteroides* spp., *Bifidobacterium* spp., *E. coli* and *Enterobacteriaceae* spp.

SCFA, short chain fatty acid; FISH, fluorescent in situ hybridization; qPCR, quantitative real time polymerase chain reaction; DGGE, denaturing gradient gel electrophoresis; KEGG, Kyoto Encyclopedia of Genes and Genomes; OTU, operational taxonomic unit;; ↑, increased; ↓, decreased; ↔, no change

Figure 1. A) Variations in microbial numbers across the length of the GIT B) Selected features affecting microbiota establishment and maintenance and factors influencing microbial composition. Microorganisms are listed where their abundance is related to a particular environmental factor (Arumugam *et al.*, 2011; Cusack & O'Toole, 2010; Dominguez-Bello *et al.*, 2010; Isolauri *et al.*, 2004; Marques *et al.*, 2010; Morelli, 2008; Qin *et al.*, 2010; Tap *et al.*, 2009; Tiihonen *et al.*, 2010).

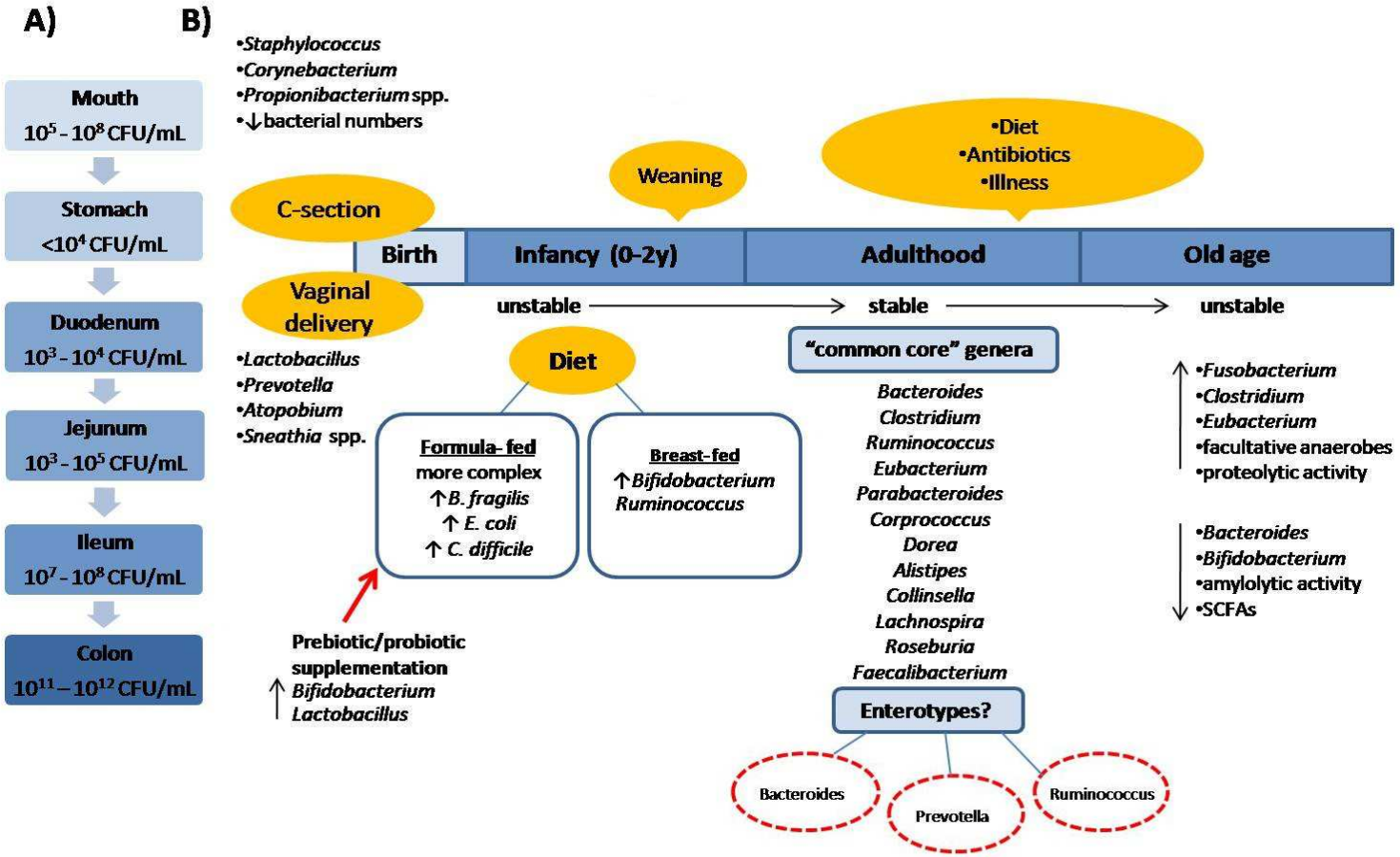
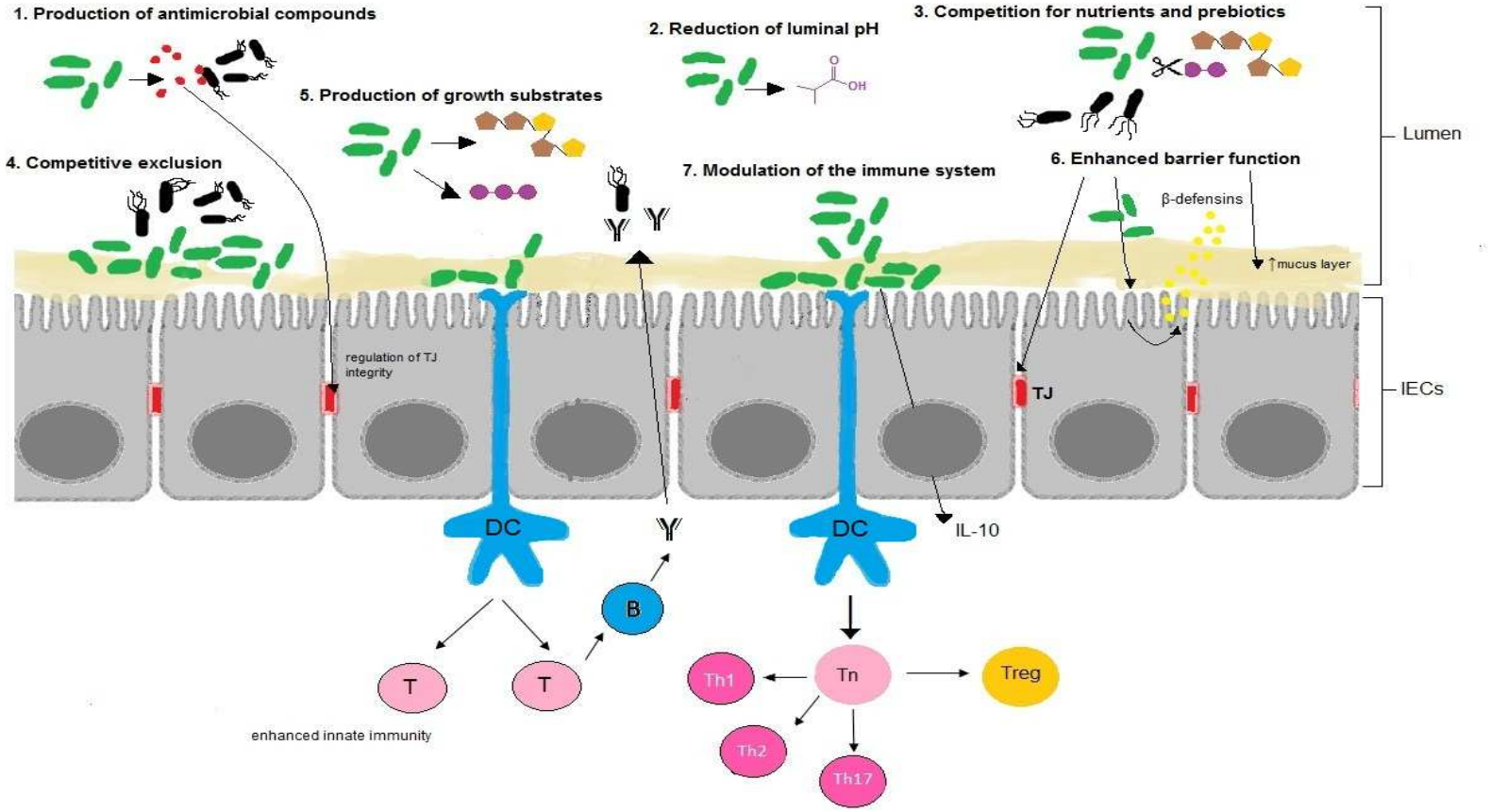


Figure 2. Schematic diagram illustrating selected mechanisms by which probiotic bacteria may influence the intestinal microbiota and/or induce beneficial host responses: 1) production of antimicrobial compounds (e.g. bacteriocins); 2) reduction of luminal pH through production of short-chain fatty acids (SCFA); 3) competition with pathogens for nutrients and prebiotics; 4) competitive exclusion of pathogens for adhesion to epithelial cells; 5) production of growth substrates (e.g. vitamins, SCFAs and exopolysaccharide); 6) enhanced intestinal barrier function (e.g. increased mucus and β-defensin secretion and/or modulation of cytoskeletal and tight junctional protein phosphorylation and; 7) modulation of immune response. IEC: intestinal epithelium cells; DC: dendritic cell; TJ: tight junction (modified from O'Toole & Cooney, 2008)



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CHAPTER 2

Intestinal microbiota, obesity and metabolic syndrome

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1.0 Abstract

The relatively recent discovery that changes in the composition and metabolic activity of the gut microbiota are associated with obesity and related disorders has led to an explosion of interest in this now distinct research field. In the following chapter, we discuss the current evidence related to how the modulation of gut microbial populations might have beneficial effects with respect to controlling obesity. A number of studies in both animals and humans have shown that the composition of the gut microbiota is significantly altered in obesity and diabetes. Strategies including specific functional foods, probiotics and prebiotics have the potential to favourably influence host metabolism by targeting the gut microbiota. Indeed, probiotics appear to be a promising approach to alter the host metabolic alterations linked to the changes in the gut microbiota. However, the mechanisms by which probiotics may impact on the development of obesity and metabolic health remain unclear and require further investigation.

2.0 Introduction

The prevalence of obesity and its associated metabolic disorders has increased substantially over recent decades to a point where they have reached epidemic levels worldwide. As such, obesity is a major public health issue and is associated with an increased risk of cardiovascular disease (CVD), type 2 diabetes mellitus (T2D), atherosclerosis, non-alcoholic fatty liver disease (NAFLD) and certain cancers. Obesity is a complex condition that results from an imbalance between energy intake and expenditure and appears to be influenced by a combination of genetic, lifestyle and environmental factors. In this chapter the current evidence linking gut microbiota with the development of obesity and obesity-related metabolic diseases and the effects of interventions, and probiotics, in particular, on the management and prevention of obesity will be discussed.

3.0 Gut microbiota and obesity

3.1 Animal Studies

The availability of animal models and the advent of new molecular, culture-independent techniques have together greatly enhanced our ability to study the gut microbiota and its relationship to obesity. Mice, in particular, offer a suitable model for conducting such experiments as their gut microbiota, by virtue of a predominance of *Firmicutes* (60-80%) and *Bacteroidetes* (20-40%), exhibits an overall pattern that is similar to that of humans (Ley *et al.,* 2005). Furthermore, germ-free (GF) mice are particularly useful for understanding the role of the gut microbiota in health and disease and the mechanisms underlying microbiota-associated alterations in host physiology. A landmark study by Bäckhed *et al.* (2004) found that GF mice were leaner than their conventional (CONV) counterparts, even though they consumed more food. In addition, colonisation of GF mice with the gut microbiota harvested from CONV mice led to an increase in total body fat despite a related decrease in food intake. GF mice have also been shown to be protected against obesity while consuming a western-style, high-fat diet (Bäckhed *et al.,* 2007; Fleissner *et al.,* 2010) but the source and type of fat and carbohydrate may be an important factor (Fleissner *et al.,* 2010). Indeed, Fleissner *et al.* (2010) observed that GF mice fed a high-fat diet lacking sucrose, were no longer protected against the

development of diet-induced obesity, illustrating the complexity of the interactions between diet, microbes and host adiposity.

There is mounting evidence that it is not simply the presence, but also the relative proportions of the major microbial divisions, within the microbiota that are associated with obesity. The development of obesity is associated with an increase in the *Firmicutes* to *Bacteroidetes* ratio in both diet-induced obese (DIO) (Turnbaugh *et al.*, 2008; Turnbaugh *et al.*, 2009b) and genetically obese (*ob/ob*) mice (Ley *et al.*, 2005; Turnbaugh *et al.*, 2006). Furthermore, a recent study showed that feeding a high-fat diet determined the composition of the gut microbiota independent of obesity, in mice (Hildebrandt *et al.*, 2009). In this study, Hildebrandt *et al.* (2009) showed, using the resistin-like molecule β (RELM-β)-knockout mice (which are resistant to diet-induced obesity), that the high-fat diet itself, and not the obese state, mainly accounted for the observed changes in the gut microbiota composition. Other work showed that switching from a low-fat to a high-fat diet resulted in a rapid and dramatic shift in the structure of the gut microbiota in mice in a single day (Turnbaugh *et al.*, 2009b). Murphy *et al.* (2010) investigated the effects of a high-fat diet and genetic obesity (the *ob/ob* mouse model) on the gut microbiota over time. While no significant changes in the proportions of the different microbial groups were observed in the control lean mice over the 8 week period, a progressive increase in *Firmicutes* in both the high-fat-fed and *ob/ob* mice was reported, reaching statistical significance in the former case only. Moreover, *Bacteroidetes* proportions decreased overtime in all groups but again reached statistical significance in only the *ob/ob* mice. These studies suggest that microbial adaptation to diet over time, and perhaps with age, is an important variable in the complex relationship between the composition of the microbiota and obesity and should be considered in future studies.

3.2 Human Studies

In man the relationship between the gut microbiota and obesity is somewhat more unclear. The first study examining the qualitative changes of the gut microbiota in 12 human obese individuals was published by Ley and colleagues in 2006. In agreement with results from animal studies, Ley *et al.* (2006) observed an increased ratio of *Firmicutes* to *Bacteroidetes* in obese individuals compared to matched lean individuals. Interestingly, after weight loss (following a fat-restricted or carbohydrate-restricted low

calorie diet), the ratio of *Bacteroidetes* to *Firmicutes* approached a lean type profile after 52 weeks. Comparison of the faecal microbiota of monozygotic and dizygotic twins who were either lean or obese also revealed that obesity was associated with a reduced representation of *Bacteroidetes*, reduced bacterial diversity and increased proportions of *Actinobacteria*. Notably, no significant differences in the proportions of *Firmicutes* were apparent between lean and obese subjects (Turnbaugh *et al.,* 2009a). Although a similar reduction of *Bacteroidetes* in obese subjects has been confirmed in another study (Armougom *et al.,* 2009), the concept that *Bacteroidetes* are reduced in obese subjects has been largely contradicted by other studies (Duncan *et al.,* 2008; Schwiertz *et al.,* 2010; Zhang *et al.,* 2009; Zupancic *et al.,* 2012).

Duncan *et al.* (2008) detected no differences in *Bacteroidetes* proportions between obese and non-obese individuals and no significant changes in the proportions of *Bacteroidetes* were detected in obese subjects following weight loss. Furthermore, Schwiertz *et al.* (2010) reported a decrease in the ratio of *Firmicutes* to *Bacteroidetes* in obese human adults compared with lean controls. Similarly, Zhang *et al.* (2009) reported that overweight individuals harboured more *Bacteroidetes* than normal weight individuals. They showed that a subgroup of *Bacteroidetes* (*Preveotellaceae*) was significantly enriched in obese individuals. Moreover, surgical treatment for these morbidly obese subjects (gastric bypass) altered the gut microbiota toward an increase in *Gammaproteobacteria* (members of *Enterobacteriaceae*) and a proportional decrease in *Firmicutes*. More recently, Zupancic *et al.* (2012) found no association between the *Bacteroidetes/Firmicutes* ratio and any metabolic syndrome trait in an Old Order Amish sect. However, 22 bacterial species were found to be either positively or inversely correlated with obesity and metabolic syndrome traits, indicating that specific members of the gut microbiota may play a role in these metabolic derangements. The conflicting results observed in these human studies emphasise the complexity of the relationship between gut microbiota and obesity. Further work is required to identify if obesigenic components of the gut microbiota exist. Moreover, the potential influence of confounding factors such as diet highlights the need for well-controlled large, clinical trials to identify specific proximate microbiota-related biomarkers of risk for obesity and metabolic dysregulation.

3.3 Mechanisms linking the gut microbiota to obesity

3.3.1 Host factors

The gut microbiota may be involved in the regulation of fat storage and composition. *Fiaf* (fasting induced adipocyte factor) is involved in regulating fat storage by inhibiting lipoprotein lipase, while also promoting fatty acid release by inducing peroxisomal proliferator-activated receptor (PPAR) coactivator (Bäckhed *et al.,* 2004). It has been suggested that germ-free mice are resistant to obesity through a mechanism involving *Fiaf* in the intestine (Bäckhed *et al.,* 2004). Indeed, germ-free *Fiaf* knock out (*Fiaf* -/-) mice gained significantly more weight than their germ-free wild type littermates when fed a Western diet (Bäckhed *et al.,* 2007) However, a more recent study found that while intestinal *Fiaf* mRNA was increased in germ-free mice, there was no increase in plasma protein levels compared to CONV mice (Fleissner *et al.,* 2010) suggesting that the *Fiaf* mechanism may not be universally associated with intestinal microbiota-related adiposity. Other work has suggested that the gut microbiota and its products affect host energy regulation acting through mechanisms involving adenosine monophosphate-activated protein kinase (AMPK) (Bäckhed *et al.,* 2007). Furthermore, the microbiota may also influence food intake and energy expenditure of the host (Bäckhed *et al.,* 2004; Fleissner *et al.,* 2010; Rabot *et al.,* 2010). These studies suggest that a number of host-related factors may be involved in the regulation of adiposity by the gut microbiota.

3.3.2 Energy harvesting

Colonisation of germ-free mice with an ‘obese microbiota’ (i.e. microbiota from an obese animal) leads to a significantly greater increase in adiposity compared to germ-free mice colonised with a ‘lean microbiota’ (Turnbaugh *et al.,* 2008; Turnbaugh *et al.,* 2006). Indeed, the gut microbiota may affect obesity by increasing energy harvesting from indigestible polysaccharides. It is estimated that around 20-60g of undigested dietary carbohydrates reach the colon on a daily basis (Flint *et al.,* 2012) where they are broken down by the gut microbiota to short-chain fatty acids (SCFAs), thereby contributing energy to the host (estimated to account for 5- 10% of daily energy intake) (McNeil, 1984). Metagenomic analysis of *ob/ob* caecal microbiota revealed that this obese microbiome had an increased capacity to harvest energy from the diet as it was

enriched with bacterial genes encoding enzymes for the utilisation and fermentation of dietary fibres (Turnbaugh *et al.,* 2006). Moreover, the *ob/ob* caecum had increased concentrations of the major fermentation end-products butyrate and acetate, and *ob/ob* mice had significantly less energy remaining in their faeces relative to their lean counterparts. *ob/ob* mice have also been shown to harbour more methanogenic *Archaea*, which would be expected to increase the efficiency of bacterial fermentation by removing one of its end products, H_2 (Turnbaugh *et al.,* 2006). Indeed, co-colonisation of germ-free mice with *Methanobrevibacter smithii* (the main methanogenic archaeon in the human intestine) and *Bacteroides thetaiotaomicron* (a major saccharolytic member of the human intestine) increased polysaccharide fermentation efficiency and adiposity compared with mice colonised with either organism alone (Samuel & Gordon, 2006). However, age may be an important factor in determining the potential of the gut microbiota to extract energy. Indeed, although *ob/ob* mice had less faecal energy and higher caecal concentrations of SCFAs compared to lean mice at 7 weeks of age, these patterns did not persist to the ages of 11 and 15 weeks (Murphy *et al.,* 2010). In humans, SCFA levels were shown to be significantly increased and the proportion of individual SCFA changed in favour of propionate in obese individuals compared to their lean counterparts (Schwiertz *et al.,* 2010). A recent study reported that an altered nutrient load induced rapid changes in the composition of the human gut microbiota which were directly associated with stool energy loss in lean individuals. In this case, a 20% increase in *Firmicutes* and a corresponding decrease in *Bacteroidetes* were associated with an increased energy harvest of approximately 150kcal (Jumpertz *et al.,* 2011).

3.3.3 Inflammation

Recent research has suggested that altered function of the innate immune system may promote the development of metabolic syndrome through a mechanism involving the gut microbiota. Obesity is generally associated with low grade, chronic, systemic inflammation (Wellen & Hotamisligil, 2005). Indeed, in murine models of obesity (genetic and diet-induced), the adipose tissue exhibits increased expression and content of proinflammatory cytokines (Hotamisligil *et al.,* 1993; Weisberg *et al.,* 2003). Cani *et al.* (2007b) demonstrated that excess dietary fat facilitates the absorption of the highly proinflammatory bacterial component lipo-polysaccharide (LPS), a toll-like receptor-4

(TLR-4) ligand, from the intestine. Mice fed for four weeks a high-fat diet had plasma LPS levels 2 or 3 times higher than normal, which was defined as 'metabolic endotoxemia'. Indeed, the direct infusion of LPS mimicked the physiological effects of the high-fat diet. Moreover, the effects of the high-fat diet were ameliorated in mice lacking cluster of differentiation 14 (CD14), a component of the TLR-4 receptor complex (Cani *et al.,* 2007b). In a follow-up study, the gut microbiota was implicated in metabolic endotoxaemia through the use of oral antibiotics which significantly decreased the levels of gut bacteria and systemic metabolic endotoxaemia (Cani *et al.,* 2008). In addition, while plasma LPS concentrations correlated negatively with *Bifidobacterium* spp. (Cani *et al.,* 2008), feeding mice a prebiotic (oligofructose) restored the number of bifidobacteria and reduced the impact of high-fat diet-induced metabolic endotoxemia (Cani *et al.,* 2007a). This was associated with a reduction in gut permeability, improved tight junction integrity and increased expression of glucagon-like peptide 2 (Glp2) (Cani *et al.,* 2009).

3.3.4 SCFAs

In addition to their other physiological roles, recent data suggests that SCFAs can have a role as signalling molecules (Samuel *et al.,* 2008) by acting as ligands for at least two G protein-coupled receptors (GPR41 and GPR43) on gut enteroendocrine cells (Bjursell *et al.,* 2011; Samuel *et al.,* 2008). A recent study has shown that GPR43 knock-out mice are protected from high-fat diet-induced obesity and its consequences on glucose and lipoprotein metabolism (Bjursell *et al.,* 2011). Moreover, conventional GPR41 knock-out (GPR41 -/-) mice and germ-free GPR41-/- mice colonised with a model fermentative community (*B. thetaiotaomicron* and *M. smithii)* were significantly leaner than their wild type counterparts, despite similar food intakes. However, there was no difference between germ-free wild type and germ-free GPR41-/- mice indicating that the effects of GPR41 are dependent on the gut microbiota. Moreover, GPR41 deficiency was also associated with reduced expression of peptide YY (PYY), an enteroendocrine cell-derived hormone that normally inhibits gut motility, reduces intestinal transit rate and increases harvest of energy (SCFAs) from the diet (Samuel *et al.,* 2008). Therefore these studies support the theory that intestinal metabolites (SCFAs) may also act as metabolic regulators in the host.

3.3.5 Flagellum and TLR-5

TLR-5 is highly expressed in the intestinal mucosa and is involved in arbitrating immune responses through recognition of bacterial flagellum. Vijay-Kumar *et al.* (2010) reported that TLR-5–knock-out (T5KO) mice exhibited hyperphagia and demonstrated many of the features associated with the metabolic syndrome including increased body mass and visceral fat deposition, dyslipidemia, hypertension and insulin resistance. Moreover, these metabolic changes correlated with changes in the composition of the gut microbiota which were mainly at a species rather than phylum level. Indeed, transplantation of the microbiota from T5KO mice to wild-type germ-free mice conferred many aspects of the T5KO phenotype including hyperphagia, obesity, hyperglycaemia and insulin resistance (Vijay-Kumar *et al.,* 2010).

In summary, studies suggest that gut microbiota and their metabolic products can influence obesity but that the relationship between diet, the gut microbiota and obesity is complex. Changes in diet not only leads to obesity by caloric intake *per se*, but also affect the composition of the gut microbiota and which may in turn lead to obesity through several mechanisms (e.g. regulation of energy balance and low grade inflammation). The question as to whether specific populations are responsible for obesity or just indicators (i.e., are these populations a ‘cause’ or an ‘effect’) remain unanswered. Taken together, these findings indicate that therapeutic manipulation of the microbiota may be a useful strategy in the prevention or management of obesity.

4.0 Gut microbiota and obesity-related metabolic disease

The role of the gut microbiota in obesity has been studied in detail and the majority of the published literature describes the disparities between gut microbiota in obese compared to lean subjects. Type II diabetes, cardiovascular disease and non-alcoholic fatty liver disease (NAFLD) are generally considered as consequences of obesity. Though relationships between the microbiota and these disorders are less well studied than in obesity, *per se,* inferences regarding the role of the gut microbiota in these obesity-related conditions are starting to be made.

4.1 Gut microbiota and Type 2 Diabetes

Low-grade inflammation is a common feature of type II diabetes (T2D) (Wellen & Hotamisligil, 2005) and LPS is a possible initiator of metabolic impairment (Cani *et al.*, 2007b). Indeed subjects with T2D have higher LPS levels than subjects without diabetes (Creely *et al.*, 2007; Pussinen *et al.*, 2011) and the infusion of LPS in mice induces low-grade chronic inflammation and features of early onset of metabolic diseases such as visceral fat deposition, glucose intolerance and hepatic insulin resistance (Cani *et al.*, 2007b). Moreover, the development of T2D in mice was associated with increased endotoxaemia along with increased gut permeability and systemic/adipose tissue inflammation (Serino *et al.*, 2012).

However, it has been shown that not all genetically identical mice develop diabetes when fed a high-fat, carbohydrate-free diet, indicating that the metabolism of each phenotype is different despite identical genetic backgrounds and diets (Burcelin *et al.*, 2002; Serino *et al.*, 2012). Serino *et al.* (2012) identified a gut microbial profile specific to diabetes-sensitive and diabetes-resistant metabolic phenotypes and found that an increased *Bacteroidetes* to *Firmicutes* ratio and a reduction in *Lachnospiraceae* family and *Oscillibacter* genus were associated with the diabetic phenotype. Furthermore, modulation of the gut microbiota of these high-fat fed mice with dietary fibres prevented the occurrence of the diabetic phenotype and resulted in a specific microbial signature (Serino *et al.*, 2012). Similarly, modulation of the gut microbiota with antibiotics has been shown to reverse insulin resistance in *ob/ob* mice independent of obesity (Membrez *et al.*, 2008); while it has also been demonstrated that germ-free mice are resistant to high-fat, diet-induced insulin resistance (Rabot *et al.*, 2010).

A small number of human, clinical studies have also reported on the relationship between gut microbiota composition and T2D. Indeed, there is increasing evidence which suggests that T2D can be associated with the gut microbiota, irrespective of the presence of obesity (Larsen *et al.*, 2010; Wu *et al.*, 2010). The proportions of phylum *Firmicutes* and the class *Clostridia* were shown to be reduced while the class *Betaproteobacteria* was enriched in diabetic subjects compared to non-diabetic controls. Interestingly, the ratios of *Bacteroidetes* to *Firmicutes,* as well as the ratios of *Bacteroides-Prevotella* group to the *Clostridium coccoides-Eubacterium rectale* group, correlated positively and significantly with plasma glucose concentration but not with

body mass index (BMI) (Larsen *et al.,* 2010). Other studies have found increased *Bacteroides* and reduced *Prevotella*, *Bifidobacterium*, *Bacteroides vulgatus* (Wu *et al.,* 2010) and *Faecalibacterium prausnitzii* proportions associated with diabetes (Furet *et al.,* 2010). Recently, deep shotgun sequencing of the gut microbial DNA from 345 Chinese individuals identified approximately 60,000 type-2-diabetes-associated markers, a decrease in the abundance of some universal butyrate-producing bacteria and an increase in various opportunistic pathogens, as well as an enrichment of other microbial functions conferring sulphate reduction and oxidative stress resistance. An analysis of 23 additional individuals demonstrated that these gut microbial markers might be useful for classifying T2D (Qin *et al.,* 2012). Furthermore, a very recent study has shown that infusion of the gut microbiota from lean donors to male recipients with metabolic syndrome for six weeks resulted in an increased insulin sensitivity of the recipients along with increased levels of butyrate-producing gut microbiota (Vrieze *et al.,* 2012). These latter two studies highlight the role of SCFA, in particular butyrate in the relationship between the gut microbiota and T2D.

4.2 Gut microbiota and cardiovascular disease

A link between the gut microbiota and cardiovascular disease (CVD) has recently been established following the discovery of a microbial-dependent pathway for the metabolism of dietary phospholipids which generates metabolites that are pro-atherosclerotic after absorption and hepatic metabolism (Wang *et al.,* 2011). The gut microbiota metabolises dietary phosphatidylcholine (present in high-fat foods) to trimethylamine (TMA), which is then transported to the liver and oxidised to trimethylamine-N-oxide (TMAO). Wang *et al.* (2011) found that increasing levels of plasma TMAO, choline and betaine was found to have dose-dependent relationships with the presence of CVD in a cohort of 1,876 men and women. Furthermore, dietary supplementation of atherosclerosis-prone mice with choline, TMAO or betaine promoted up-regulation of multiple macrophage scavenger receptors linked to atherosclerosis, and dietary supplementation with choline or TMAO promoted atherosclerosis. This study suggests opportunities for the development of novel therapeutic strategies for the treatment for atherosclerosis.

Furthermore, it has been shown that germ-free mice were resistant to high-fat, diet-induced dyslipidemia and elevated proinflammatory markers compared to their CONV

counterparts. Moreover, there were unexpected changes in cholesterol metabolism, including reduced hypercholesterolemia, a moderate increase of hepatic cholesterol, an increase in cholesterol excretion and an up-regulation of cholesterol biosynthesis gene (Rabot *et al.,* 2010).

Low-grade inflammation is a common feature of CVD (Wellen and Hotamisligil, 2005) and metabolic endotoxemia has also been linked to its development. In a 5 year prospective study (the Bruneck study) it was demonstrated that subclinical endotoxemia constitutes a strong risk factor for the development of carotid atherosclerosis, among smokers in particular (Wiedermann *et al.,* 1999).

The relationship between both the oral and gut microbiota and atherosclerosis has recently been investigated. Although no relationship was found between plaque microbiota and gut microbiota in affected patients, the abundance of certain bacteria (*Veillonella* and *Streptococcus*) in atherosclerotic plaque correlated with their abundance in the oral cavity (Koren *et al.,* 2011).

4.3 Gut microbiota and NAFLD

Although there is a relative paucity of data, the microbiota may also be linked to the development and progression of NAFLD and non-alcoholic steato-hepatitis (NASH). It has been shown that germ-free mice are resistant to high-fat diet-induced hepatic steatosis (Rabot *et al.,* 2010) and colonisation of germ-free mice with the gut microbiota harvested from CONV mice increased liver fat and liver triglyceride content (Bäckhed *et al.,* 2004). Moreover, NAFLD and NASH have been associated with increased intestinal permeability, as well as small intestinal bacterial overgrowth (SIBO) (Miele *et al.,* 2009). Several molecular mechanisms relevant to the involvement of the microbiota in NAFLD/NASH have been proposed. The increased production of SCFA by the microbiota would provide more energy to the liver (Bäckhed *et al.,* 2007; Turnbaugh *et al.,* 2006). The microbiota has also been shown to stimulate hepatic triglyceride production through suppression of *Fiaf* (Bäckhed *et al.,* 2004) and its role in choline metabolism has also been implicated as one of the mechanisms which contribute to NAFLD (Dumas *et al.,* 2006; Wang *et al.,* 2011). As liver disease is associated with a state of inflammation, LPS is also likely to be involved in NAFLD/NASH. Indeed, increased levels of LPS have been observed in NAFLD patients (Harte *et al.,* 2010).

More recently it has been shown that genetic inflammasome deficiency and its related dysbiosis result in abnormal accumulation of bacterial products in the portal circulation and drive progression of NAFLD/NASH (Henao-Mejia *et al.,* 2012).

In summary, the gut microbiota represents an important source of metabolic variability in the host and may also play a role in the development of metabolic disorders such as T2D, NAFLD and cardiovascular disease, perhaps even independently of obesity. Increased knowledge of the mechanisms involved in the interactions between the microbiota and its host will aid in the development of treatments for metabolic disease.

5.0 Probiotics: role in obesity and metabolic syndrome

The demonstration of the possible role of gut microbiota in the pathogenesis of obesity and related metabolic diseases has led to the development of different strategies to modulate the gut microbiota and hence ameliorate host metabolism. In this respect, there are differing approaches to modulate the gut microbiota. Specific bacterial species or classes may be reduced by antibiotics. Studies have shown that treatment of both *ob/ob* and DIO mice with broad spectrum antibiotics leads to changes in the composition of the gut microbiota, as well as an improvement in metabolic abnormalities including improved insulin resistance, fasting glycaemia, glucose tolerance and a reduction in both metabolic endotoxaemia and body weight gain (Cani *et al.,* 2008; Membrez *et al.,* 2008; Murphy *et al.,* 2012).

In contrast to reducing specific bacterial species through the use of antibiotics, certain beneficial bacteria may be increased either by pre- or probiotics. As discussed previously, Cani *et al.* (2007a) found that the number of caecal *Bifidobacterium* spp. was inversely correlated with the development of fat mass and glucose intolerance as well as LPS levels in high-fat diet fed mice. Prebiotics containing oligofructose were found to completely restore *Bifidobacterium* species, normalise plasma endotoxin levels as well as leading to improved glucose tolerance and glucose induced insulin secretion when administered to high-fat fed mice (Cani *et al.,* 2007a). Similarly, Serino *et al.* (2012) recently showed that the prebiotic, α1-2-gluco-oligosaccharide, reversed most aspects of the high-fat induced diabetic phenotype in mice.

The application of probiotics as a potential therapy in the management of obesity and its associated metabolic disorders has also received particular attention and has been explored in a number of studies.

5.1 Animal Studies

A number of experimental studies suggest that the probiotic approach can be used to alter biomarkers of metabolic disease (summarised in Table 1). Indeed, specific *Lactobacillus* and *Bifidobacterium* strains have been shown to reduce body weight and fat mass (Aronsson *et al.,* 2010; Ji *et al.,* 2012; Kang *et al.,* 2010; Kondo *et al.,* 2010; Lee *et al.,* 2006; Lee *et al.,* 2007; Takemura *et al.,* 2010; Tanida *et al.,* 2008) and alter lipid profiles (Hamad *et al.,* 2009; Kondo *et al.,* 2010; Martin *et al.,* 2008; Xiao *et al.,* 2003; Yadav *et al.,* 2007) in murine models, although the effect is likely to be strain dependent (Yin *et al.,* 2010). For example, milk products fermented with *Lb. gasseri* SBT2055 were shown to have a possible role in the regulation of adipose tissue growth by reducing adipocyte size, serum leptin (Sato *et al.,* 2008), fatty acid absorption (Hamad *et al.,* 2009); possibly, through an anti-inflammatory mechanism (Kadooka *et al.,* 2011). In contrast, other work has shown an increase in body weight with the administration of *Lb. fermentum* (CIP 102980) to chickens (Khan *et al.,* 2007). Indeed there has been recent debate regarding the role of probiotics in metabolic disease, prompted a proposal that these 'friendly' bacteria may play a role in the development of obesity. Raoult (2009) suggested that since probiotics are used as growth promoters in the farm industry, there is a possibility that probiotics may also promote weight gain in humans. However, this view has largely been rejected by other researchers in the field (Delzenne & Reid, 2009).

Probiotic strains may also be able to modify the fat composition of host tissues. Wall *et al.* (2009) showed that the administration of a conjugated linoleic acid (CLA)-producing *B. breve* strain, in combination with linoleic acid as a substrate, led to a modulation of the fatty acid composition of mice and pigs, including significantly elevated concentrations of hepatic and adipose tissue content of *cis-9, trans-11* CLA, omega-3 fatty acids, along with reduced levels of the proinflammatory cytokines tumour necrosis factor α (TNF-α) and interferon γ (IFN-γ).

In rat models of diabetes (high fructose-/streptozotocin–induced diabetes), specific *Lactobacillus* strains can improve such markers of diabetes as hyperinsulinemia, hyperglycemia, glucose intolerance, dyslipidemia and oxidative stress (Tabuchi *et al.*, 2003; Yadav *et al.*, 2007; Yadav *et al.*, 2008). Moreover, the probiotic product VSL#3 has been shown to improve insulin resistance, hepatic natural killer T cell depletion and hepatic steatosis in DIO mice (Ma *et al.*, 2008) while *Lb. casei* Shirota has been demonstrated to improve insulin resistance and glucose intolerance as well as reduce endotoxemia (Naito *et al.*, 2011).

5.2 Human Studies

Only a limited number of human clinical trials have assessed the effects of probiotic intake on metabolic and obesity biomarkers (summarised in Table 2).

Kadooka *et al.* (2010) carried out a double-blind, randomised, placebo controlled intervention trial among subjects (n=87) with an increased BMI and abdominal visceral fat area. Subjects received either a fermented milk product containing *Lb. gasseri* LG2055 or the same product without LG2055 over a period of 12 weeks. Abdominal fat area was determined by computed tomography. In subjects receiving the probiotic, abdominal visceral and subcutaneous fat areas decreased significantly from baseline (by an average of 4.6 and 3.3%, respectively). Body weight, BMI, hip and waist measures also decreased significantly while none of those parameters changed significantly in the control group (Kadooka *et al.*, 2010).

A large study by Laitinen *et al.* (2009) showed that the combination of dietary counselling and a probiotic intervention led to consistently improved glucose metabolism and insulin sensitivity in a cohort (n=256) of healthy, normoglycaemic pregnant women. Moreover, the probiotics were shown to provide a greater glucose-lowering effect than dietary counselling alone. In a double-blind RCT, Andreasen *et al.* (2010) allocated male subjects (n=45) with T2D, impaired or normal glucose tolerance to a 4 week treatment with either *Lb. acidophilus* NFCM or placebo. Insulin sensitivity was preserved in the *Lb. acidophilus*-fed group whereas it decreased in the placebo group. However, there was no change in inflammatory markers over the four week period indicating that this effect was not linked to the immune system.

A number of studies have also investigated the cholesterol lowering effects of probiotic strains. Bukowska *et al.* (1998) investigated the role of 'Pro viva' (a food product containing a fermentable oat fraction and the probiotic *Lb. plantarum*299v) supplementation on the atherogenic markers low density lipoprotein (LDL)-cholesterol and fibrinogen in a double blind cross-over study among 30 male subjects. Six weeks of treatment resulted in a reduction in levels of both markers (Bukowska *et al.,* 1998). In a subsequent study, Naruszewicz *et al.* (2002) investigated the influence of *Lb. plantarum* in a double-blind RCT involving 36 smokers and found that the 6 week treatment reduced systolic blood pressure along with several pro-atherogenic markers, including plasma concentrations of fibrinogen, IL-6 and F2-isoprostanes (markers of lipid oxidant stress). Moreover, *Lb. plantarum* administration markedly decreased the adherence of monocytes to native and tumor necrosis factor–activated endothelial cells (Naruszewicz *et al.,* 2002). Similarly, Kullissar *et al.* (2003) investigated the effect of a probiotic product (goats milk fermented with *Lb. fermentum* ME-3) on oxidative stress markers (including pro-atherogenic markers) in 21 healthy subjects. Consumption of the fermented goats' milk altered both the prevalence and proportion of lactic acid bacteria species in the gut microbiota, and exhibited antioxidative and anti-atherogenic effects in the healthy subjects. Furthermore, Kiessling *et al.* (2002) demonstrated in a cross-over type study including 29 women that daily consumption of a yogurt supplemented with *Lb. acidophilus* 145, *B. longum 913* and oligofructose increased serum concentration of high-density lipoprotein (HDL)-cholesterol and led to the desired improvement of the LDL/HDL cholesterol ratio compared with a control yoghurt. Xiao *et al.* (2003) investigated the effect of consuming a low-fat yoghurt containing *B. longum* BL-2 on the lipid profiles of 32 healthy males and showed a decline in serum total cholesterol after 4 weeks, particularly among subjects with moderate hypercholesterolemia.

Although many of these reports demonstrate a cholesterol-lowering effect, a number of studies have failed to show such an effect in humans. For example, Lewis & Burmeister conducted a cross-over type study in eighty healthy volunteers with elevated cholesterol and found that supplementation with *Lb. acidophilus* had no significant effect on plasma total-, LDL- and HDL-cholesterol and triglycerides (Lewis & Burmeister, 2005). Simons *et al.* (2006) also demonstrated that the consumption of *Lb. fermentum* did not contribute to any significant lipid profile changes after its administration to healthy subjects with elevated cholesterol levels for 10 weeks (Simons *et al.*, 2006). Similarly,

the administration of a synbiotic product (*Lb.acidophilus*, *B. longum* and oligofructose) for approximately 2 months had no significant effect on plasma cholesterol or triglyderides in normocholesteraemic volunteers (Greany *et al.*, 2007). These contradictory findings are probably due to differences in experimental design and study population as well as variations in probiotic strain and dosage.

Many questions still remain regarding the role of probiotic bacteria in obesity and metabolic disease and current evidence is derived mainly from animal studies. Although these results from animal studies are interesting, the underlying mechanisms are unclear. Only a minority of human studies have examined the benefits of probiotic bacteria on biomarkers of metabolic disorders and these have shown conflicting results. In addition, the majority of these trials have been relatively small-sized (< 50 subjects) and of short duration (<10 weeks). The differences between studies may arise due to the use different bacterial strains. Indeed, the results from one probiotic strain cannot be extrapolated to another. Moreover, quite often different delivery systems (e.g. fermented dairy product v freeze dried bacteria); different population groups (e.g. normocholesterolaemic v hypercholesterolaemic) as well as different administration dosages have been employed, rendering comparisons difficult.

6.0 Conclusions

Obesity and associated metabolic diseases are increasing in prevalence worldwide. Both experimental and clinical data have demonstrated that obesity is associated with changes in the gut microbiota. Nonetheless, many unanswered questions still remain. Researchers have yet to conclusively identify ‘obesigenic’ bacterial groups. Moreover, the question still remains whether the differences in the microbiota between lean and obese groups are the cause or the consequence of obesity or perhaps a combination of both. Future research is therefore challenged with the task of identifying beneficial bacteria which are capable of controlling adiposity and related metabolic disorders and also with investigating possible dietary approaches to promote these bacteria in order to improve metabolic health. Indeed, data is accumulating which suggest that manipulation of the microbiome using prebiotics or probiotics may reduce insulin resistance and fat accumulation. Although a number of animal studies have revealed promising results, it is difficult to apply these findings to man. Only a limited number of small-sized clinical trials have demonstrated the promise for specific probiotic strains in beneficially

modulating biomarkers of metabolic disease in humans. However, the potential implications of this advancing field are promising.

Table 1. Effects of probiotic consumption on biomarkers of metabolic disease in animal models

Author	Probiotic	Model	Duration	Outcome
Aronsson *et al.* (2010)	*Lb. paracasei* ssp. *paracasei* F-19 (2×10^9 CFU/g feed)	Male C57BL/6J mice-high-fat diet	10 wk	↓ body fat ↑ circulating *Fiaf*
Hamad *et al.* (2009)	Skim milk fermented with *Lb. gasseri* SBT2055 (6×10^7 CFU/g diet)	Lean and obese Zucker rats	4 wk	↓ serum total and HDL cholesterol ↑ excretion of faecal fatty acids and total neutral faecal sterols Lean rats: ↓ mesenteric adipose tissue weight ↓ adipocyte sizes ↓serum leptin Obese rats: ↑ no. of smaller adipocytes in the subcutaneous adipose tissue
	Skim milk fermented with *Lb. gasseri* SBT2055 (6×10^7 CFU/g)	Male Sprague-Dawley rats (with permanent cannulation of thoracic duct)	1 wk	↓maximum transport rate of TG and phospholipids
Ji *et al.* (2012)	*Lb. rhamnosus* GG (1×10^8 CFU/day) or *Lb. sakei* NR28 (1×10^8 CFU/d)	Male C57BL/6J mice	3 wk	↓epididymal fat ↓obesity related biomarkers such as FAS, ACC and SCD-1 from the liver
Kadooka *et al* .(2011)	Yoghurt containing *Lb. gasseri* SBT2055 (5×10^8 CFU/g)	Male Sprague Dawley rats	4 wk	inhibition of enlargement of visceral adipocytes prevention of up-regulation of sICAM-1 (inflammatory marker that is elevated in obesity)
Kang *et al.* (2010)	*Lb. gasseri* BNR17 (10^9 CFU/0.5mL - administered twice daily)	Male Sprague Dawley rats - high carbohydrate diet	12 wk	↓ weight gain ↓ fat pad mass ↔ serum total, HDL and LDL cholesterol ↔ TG
Kondo *et al.* (2010)	*Bifidobacteriumbreve* B-3 (10^8 or 10^9 CFU/d)	Male C57BL/6J mice - High-fat DIO	8 wk	↓ body weight and epididymal fat ↓ serum levels of total cholesterol, fasting glucose and insulin ↑ expression of genes related to fat metabolism and insulin sensitivity in gut and epididymal fat tissue
Lee *et al.* (2006)	*Lb. rhamnosus* PL60 (1×10^7 CFU/d or 1×10^9 CFU/d)	Male C57BL/6J mice - High-fat DIO	8 wk	↓weight gain ↓ white adipose tissue (epididymal and perirenal) ↑ apoptotic signals and UCP-2 levels in adipose tissue
Lee *et al.* (2007)	*Lb. planatarum* PL62 (1×10^7 CFU/d or 1×10^9 CFU/d)	Male C57BL/6J mice - High-fat DIO	8 wk	↓ weight of epididymal, inguinal, mesenteric and perirenal white adipose tissue ↓ blood levels of glucose ↓ body weight
Ma *et al.* (2008)	VSL#3 probiotics (mixture of bifidobacteria, lactobacilli and *Streptococcus thermophilus*) (1.5×10^9 CFU/d)	Male C57BL/6J mice - High-fat, diet induced hepatic steatosis and insulin resistance	4 wk	↑ hepatic natural killer T (NKT) cell depletion. ↓ weight improved insulin resistance improved steatosis
Martin *et al.* (2008)	*Lb. paracasei* NCC2461 or *Lb. rhamnosus* NCC4007 (1×10^8 CFU/d)	Female germ-free C3H mice - colonised with human baby flora	2 wk	↓caecal acetate and butyrate ↑caecal isobutyrate and isovalerate with *Lb. paracasei* ↓plasma LDL- and VLDL cholesterol ↑TG
Naito *et al.* (2011)	*Lb.casei* strain ShirotaYIT 9029 (heat-killed)	Male C57BL/6J mice - High-fat DIO	4 wk	improves insulin resistance and glucose intolerance. ↓endotoxaemia

Sato *et al*. (2008)	Skim milk fermented with *Lb. gasseri* SBT2055 (6 x 10^7 CFU/g diet)	Male Sprague-Dawley rats	4 wk	↓adipocyte size in mesenteric white adipose tissue ↑no. of small adipocytes from mesenteric and retroperitoneal adipose tissues ↓ serum leptin
Tabuchi *et al*. (2003)	*Lb. rhamnosus* GG	Male Wistar rats (streptozotocin induced diabetes)	9 wk	↓glycosylated haemoglobin improved glucose tolerance
Takemura *et al* . (2010)	*Lb. plantarum* LP14 (1 x 10^8 CFU/mouse-administered intragastrically)	Female C57BL/6 mice - high-fat diet	11 wk	↓ adipocyte size ↓ white adipose tissue weight ↓ serum total cholesterol ↓ serum leptin
Tanida *et al*. (2008)	*Lb. paracasei* ST11 (1 x 10^9 CFU/2mL water)	Male Wistar rats - High-fat DIO	11 wk	↓ weight gain ↓abdominal fat tissue weight
Wall *et al*. (2009)	*B. breve* NCIMB 702258 (10^9 microorganisms per day) + linoleic acid	Healthy BALB/c mice, SCID mice and weaning pigs	8 wk	↑ hepatic and adipose tissue content of cis-9, trans- 11 CLA, DHA and EPA ↓ splenocytes production of TNF-α, IL-6 and IFN-γ
Xiao *et al*. (2003)	Milk product fermented with *B. longum* BL-1 (approx 4 x 10^8 CFU/g)	Male Sprague Dawley rats	3 wk	↓ serum total and LDL cholesterol ↓TG
Yadav *et al*. (2007)	Dahi product containing *Lb. acidophilus* and *Lb. casei*	Male Wistar rats (high-fructose induced diabetes)	8 wk	↓ plasma glucose, glycosylated haemoglobin and insulin and liver glycogen ↓ total, LDL and VLDL cholesterol ↓TG and free fatty acids ↓thiobarbituric acid-reactive substances ↑reduced glutathione in liver and pancreas
Yadav *et al*. (2008)	Dahi product containing *Lb. acidophilus* and *L. casei*(7.3 x 10^9 CFU/g - 15g/day/rat)	Male Wistar rats (streptozotocin induced diabetes)	4 wk	↓ oxidative damage in pancreatic tissues by inhibiting the lipid peroxidation and formation of nitric oxide preserved antioxidant pool such as glutathione content and activities of super oxide dismutase, catalase and glutathione peroxidase
Yin *et al*. (2010)	*Bifidobacteria* strains - L66-5, L75-4, M13-4 and FS31-12	Male Sprague Dawley rats-high-fat diet	6 wk	the response to energy metabolism was strain dependent: M13-4 ↑body weight gain L66-5 ↓body weight gain all 4 strains ↓ serum and liver TG

ACC, acetyl CoA carboxylase; CFU, colony forming units; CLA, conjugated linoleic acid; DHA, docosahexanoic acid; DIO, diet induced obesity; EPA, eicosapentanoic acid; FAS, fatty acid synthase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; IL-6, interleukin 6 ;IFN-γ, interferon-γ; SCD-1, stearoyl-CoA desaturase-1; SCID, severe combined immunodeficient; sICAM-1,soluble intercellular adhesion molecule-1; TG, triglycerides; UCP-2, uncoupling protein-2; VLDL, very low density lipoprotein

Table 2. Effects of probiotic consumption on biomarkers of metabolic disease in human subjects

Author	Probiotic	Experimental design	Subjects (mean age)	Duration	Outcome
Andreasen *et al.* (2010)	*Lb. acidophilus* NCFM (1 x 10^{10} CFU/d)	Double-blind RCT - 2 arms 1) probiotic 2) placebo	45 males T2D, impaired or normal glucose tolerance (mean age 55.6 yrs)	4 wk	Insulin sensitivity was preserved in the probiotic group but ↓ in the placebo group ↔ inflammatory markers or systemic immune response in either group.
Bukowska *et al.* (1998)	Pro viva – a food product containing fermentable oatmeal with *Lb. plantarum* 299v (~ 5 x 10^7 CFU/mL - 200mL administered daily)	Double-blind RCT -2 arms 1) Pro viva product 2) Rose-hip drink (control)	30 healthy males with moderately elevated serum cholesterol (mean age 42.6 yrs)	6 wk	↓ total and LDL-cholesterol ↓fibrinogen ↔ Triglycerides, HDL-cholesterol, glucose levels and BMI
Greany *et al.* (2007)	*L. acidophilus* DDS-1, *B. longum* UABL-14 (10^9 CFU) and oligofructose (10-15g) per capsule - administered 3 times a day	Single-blind RCT- 2 arms 1) probiotic 2) placebo	55 normacholesterolemic males and females (age range18-36 yrs)	2 months (males) or 2 menstrual cycles (females)	↔ plasma concentrations of total cholesterol, HDL cholesterol, LDL-cholesterol and triglycerides
Kadooka *et al.* (2010)	Fermented milk containing *Lb. gasseri* SBT2055	Double-blind RCT -2 arms 1) fermented milk + probiotic 2) fermented milk	87 subjects with an increased BMI (24.2-30.7 kg/m^2) and abdominal visceral fat area (81.2-178.5 cm^2).	12 wk	↓abdominal visceral and subcutaneous fat areas ↓ body weight, BMI, hip and waist measurements
Kiessling *et al.* (2002)	Yoghurt containing *L. acidophilus* 145 (10^6-10^8 CFU/g), *B. longum*913 (> 10^5 CFU/g) and 1% oligofructose - 300g administered daily)	Cross-over study (7 week period each) 1) control (for all women) 2) & 3) control or probiotic	29 healthy females – including normacholesterolemic and hypercholesterolemic subjects (mean age 34 yrs)	7 wk	↑ HDL cholesterol ↓ LDL/HDL cholesterol ratio. ↔ total cholesterol and LDL-cholesterol
Kullisar *et al.* (2003)	Goats milk fermented with *Lb. fermentum* ME-3 (3 x 10^{11} CFU/d)	CT – 2 arms 1)Goats milk + probiotic (n=16) 2) Goats milk (control; n=5)	21 healthy males and females (mean age 50 yrs)	3 wk	enhanced total antioxidative activity prolonged resistance of the lipoprotein fraction to oxidation ↓ levels of peroxidised lipoproteins, oxidised LDL and 8-isoprostanes ↓glutathione redox ratio
Laitinen *et al.* (2009)	*Lb. rhamnosus* GG and *B. lactis*Bb12 (1 x 10^{10} CFU/d each)	Double-blind RCT - 3 arms 1) Diet/Probiotics 2)Diet/Placebo 3) Control/placebo	256 healthy pregnant females (mean age 30 yrs)	from1st trimester to the end of exclusive breastfeeding	fasting plasma glucose, serum insulin and HOMA were lowest in the diet/probiotic group during 3rd trimester of pregnancy and 12 month post partum
Lewis *et al.* (2005)	*L. acidophilus* LA-1 (6 x 10^{10} CFU - 3 times a day)	Cross-over study (6 week period each) - 1) probiotic 2) control	80 healthy males and females with elevated cholesterol (mean age 47 yrs)	6 wk	↔ plasma lipids

Naruszewicz *et al.* (2002)	Rose-hip drink containing *Lb. plantarum*299v (5×10^7 CFU/mL - 400mL administered daily)	Double-blind RCT - 2 arms 1) Rose-hip drink + probiotic 2)Rose-hip drink	36 healthy male and female smokers (mean age 42.3 yrs)	6 wk	↓systolic BP and fibrinogen ↓ F2-isoprostanes and IL-6 isolated monocytes showed significantly reduced adhesion to native and stimulated endothelial cells
Simons *et al.* (2006)	PCC® *Lb. fermentum*(2×10^9 CFU/capsule - 2 capsules administered twice daily)	Double-blind RCT - 2 arms 1) PCC® Lb. fermentum 2) placebo	46 healthy males and females with elevated serum cholesterol (mean age 51.5 yrs)	10 wk	LDL cholesterol showed a modest downward trend for both probiotic and placebo no significant difference in other measurements between treatment arms (total cholesterol, HDL cholesterol, triglycerides
Xiao *et al*. (2003)	Low-fat yoghurt containing *B. longum* BL-1 (approx 10^8 CFU/mL - 300mL administered daily)	Single-blind RCT - 2 arms 1) Low fat-yoghurt + probiotic 2) Low-fat yoghurt	32 healthy males with serum cholesterol ranging from 220-280 mg/dl (mean age 43.8 yrs)	4 wk	↓serum cholesterol in half of subjects, particularly among subjects with moderate hypercholesterolemia (serum total cholesterol >240mg/dl)

BMI, body mass index; BP, blood pressure; CFU, colony forming units; CT, controlled trial; HDL, high-density lipoprotein; HOMA, homeostatic model assessment(method used to quantify insulin resistance and beta-cell function); LDL, low-density lipoprotein; RCT, randomised controlled trial; T2D, type 2 diabetes mellitus; ↓, decreased; ↑, increased; ↔, unchanged

6.0 References

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CHAPTER 3

Food and nutrient intake of Irish community-dwelling elderly subjects: who is at nutritional risk?

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1.0 Abstract

The global proportion of older persons is increasing rapidly. Poor dietary habits and inadequate nutrient intakes are of particular concern in this population group and may influence quality of life and health outcomes. The aim of the present study was to assess the dietary intakes of Irish community-dwelling elderly individuals, participating in the ELDERMET project. Two hundred and eight (94 males, 114 females) subjects aged 64-93 yrs were analysed. Dietary intake was assessed using a validated semi-quantitative food frequency questionnaire (FFQ). A high rate of overweight/obesity was observed in this population group. Consumption of energy-dense, low-nutrient foods was excessive. Older elderly subjects (≥75 yrs) consumed significantly (P<0.01) more desserts/sweets than younger elderly (64-74 yrs). Intakes of dietary fat and saturated fat were high while dairy food consumption was inadequate in both males and females. Elderly females typically had a more nutrient dense diet than males. A considerable proportion of subjects, particularly males, had inadequate intakes of calcium, magnesium, vitamin D, folate, zinc and vitamin C. The data indicate that the diet of Irish community-dwelling elderly individuals is sub-optimal with respect to nutrient intake, and excessive in terms of fat intake, with implications for the health status of this population group. Reductions in dietary fat and increased low fat dairy food intakes are recommended for the prevention of diet-related disease in older persons. In addition, strategies to improve a number of sub-optimal micronutrient intakes need to be developed and implemented, particularly among elderly Irish males.

2.0 Introduction

Older persons represent an increasing proportion of world populations. In the period 2010-2050, the number of people aged 65 yrs or over is projected to increase from 8% to 16% of the world population (World Health Organisation/National Institute of Health, 2011). In 2011, approximately 12% of the inhabitants of the Republic of Ireland were aged 65 years or over (Central Statistics Office, 2012), a figure that is predicted to rise to 22% by the year 2041 (McGill, 2010). This dramatic increase in the proportion of older citizens can partly be attributed to the improved living conditions and health services of modern society (Department of Health, 2012; McGill, 2010). An increasingly elderly population poses a major challenge to public healthcare systems and to exchequer funds due to an increased prevalence of age-related chronic diseases (Christensen *et al.,* 2009; Gariballa & Sinclair, 1998) but also due to an increased demand for long-term care services (Health Service Executive, 2009; World Health Organisation/National Institute of Health, 2011; Wren *et al.,* 2012). Evidence suggests that several age-onset chronic diseases including cardiovascular disease, cancer and osteoporosis are influenced by diet (World Health Organisation, 2003; Fontana, 2009). Improving dietary intakes among the elderly would undoubtedly improve health, quality of life and reduce mortality (Anderson *et al.,* 2011; Bamia *et al.,* 2007; Iimuro *et al.,* 2012).

A number of key factors contribute to malnutrition in the elderly including inadequate dietary intakes, increased metabolic demands or malabsorption of nutrients (Chen *et al.,* 2008; Corish & Kennedy, 2000). In addition, a decrease in olfactory functioning including decreased perception of taste and smell, dental condition, disease, medications, anorexia, disability and reduced physical activity, depression, bereavement, economic instability and dementia (Brownie, 2006; Mila *et al.,* 2012) can all have a negative impact on dietary intakes among elderly individuals. Malnutrition is frequently associated with several adverse health outcomes associated with ageing, impaired quality of life and increased morbidity and mortality (Chen *et al.,* 2008; Lundin *et al.,* 2012). Nevertheless the prevalence of overweight and obesity is increasing in all population groups (James *et al.,* 2001), including the elderly (Andreyeva *et al.,* 2007) and is strongly associated with chronic disease risk and poor health outcomes. Older persons have much lower energy requirements than younger

subjects which can be attributed to changes in body composition (decrease in lean body mass and increase in body fat) and reduced physical activity (Brownie, 2006; Chernoff, 2005; Elmadfa & Meyer, 2008). Despite a reduction in energy requirements, micronutrient requirements for most vitamins and minerals remain similar to those recommended for adult populations (Elmadfa & Meyer, 2008) with requirements for some micronutrients (e.g. calcium) increased (Institute of Medicine, 2011). Good food choices and a high quality, varied diet therefore become extremely important determinants of optimal health with advancing years (Brownie, 2006).

The vast majority (94%) of elderly subjects in Ireland are currently living in the community (Central Statistics Office, 2012). Although life expectancy in Ireland has increased significantly over recent decades, a lower proportion of these years are spent disability-free in comparison to other European countries such as Sweden, Malta and Bulgaria (European Joint Action on Healthy Life Years, 2012). Because inadequate nutritional status can be an obstacle to healthy ageing (Amarantos *et al.,* 2001; Ferry & Roussel, 2011) and nutritional adequacy may prevent adverse health outcomes and significantly delay the need for long-term residential care, it is important to identify individuals who are at risk of over- or under- consumption of specific nutrients.

The ELDERMET consortium (http://eldermet.ucc.ie) was established in 2007 to investigate how the intestinal bacteria influence, and are influenced by, diet, health and lifestyle in 500 older Irish people, across a range of health profiles and including those living in the community, day-hospital attendees, rehabilitation patients and long-term care residents. This project has previously elucidated links between microbiota composition, diet, health and frailty status in the elderly (Claesson *et al.,* 2011; Claesson *et al.,* 2012). The present study was based on the hypothesis that different subgroups within the community-dwelling elderly population may be at nutritional risk. The aim of this study was therefore to assess dietary intakes among an Irish community-dwelling elderly population group who participated in the ELDERMET project and to establish their compliance with macro- and micronutrient recommendations.

3.0 Materials and Methods

3.1 Subject Recruitment

The present study investigates dietary intakes among a sub-set of self-selected, community-dwelling ELDERMET participants (*n* 208; 94 males, 114 females; 64-93 years), who responded to advertising in local health-care and general practice centres, active retirement groups and events, information sessions and local media from the Cork city and county region of southern Ireland. Community-dwelling, day-hospital attendees, were also included in the present study. Eligible candidates were community-dwellers (i.e. not in residential care such as nursing homes/long-term care facilities), aged 64 years and older and in any stage of health. Exclusion criteria included history of alcoholism, participation in investigational medication trials in the previous month and advanced organic disease (precluding longitudinal follow-up). Two subjects were excluded from the analyses due to unrealistic reported values for daily energy intakes (>4000 kcal).

3.2 Ethical Considerations

Written informed consent was obtained from all subjects in accordance with local Clinical Research Ethics Committee of the Cork Teaching Hospitals. This study complies with the guidelines set out in the Declaration of Helsinki and those of the Cork Research Ethics Committee, Ireland.

3.3 Data Collection

Height and weight were recorded by the research nursing personnel. Height was predicted from forearm (ulna) length (Mitchell & Lipschitz, 1982) which was measured to the nearest 0.5 centimetre using a tape measure. Weight was measured in light clothing to the nearest 0.1kg. All subjects were able to complete the measurements (step on scale, keep body straight, etc.). Body Mass Index (BMI) was calculated as weight (kg)/height (m^2); subjects were classified into four BMI categories according to World Health Organisation (WHO) recommendations (World Health Organisation, 2013). The Mini Nutritional Assessment (MNA) was used as a screening tool to identify subjects at risk of malnutrition (Cuervo *et al.,* 2008; Vellas *et al.,* 1999). Information on smoking status was also collected.

3.4 Dietary data

Dietary data were collected by means of a semi-quantitative food frequency questionnaire (FFQ) administered by trained research nursing personnel. The FFQ was an amended version of that used by the European Prospective Investigation into Cancer (EPIC) study (Riboli & Kaaks, 1997) and validated for use in the Irish population (Harrington *et al.,* 2008). The questionnaire assessed habitual dietary intakes of 147 single food items/beverages. The frequency of intake was measured using ten categories ranging from 'never' to 'six times a day or more'. In order to estimate the number of servings from each food group consumed on a daily basis, each frequency option in the FFQ was converted to a single daily serving (i.e. 0 for 'never', 0.01 for 'less than once a month', 0.03 for 'once a month', 0.14 for 'once a week', 0.29 for 'twice a week', etc.). Recently derived, population-based, gender-specific portion sizes for community-dwelling elderly subjects (65-75 yrs and ≥75 yrs) were applied to each FFQ item (Wrieden & Barton, 2006). Food and beverage items in the questionnaire were aggregated into 30 mutually exclusive food groups based on nutrient profiles, as described elsewhere (Leite *et al.,* 2003; Pryer *et al.,* 2001a; Pryer *et al.,* 2001b; Villegas *et al.,* 2004). Information on vitamin/mineral supplement use (including. supplement type, brand and formulations) was also collected as part of the FFQ.

To evaluate food group intakes, we took as reference the recommended number of daily servings for adults (aged 51+ yrs) from the recently revised Food Safety Authority of Ireland (FSAI) Irish food-based dietary guidelines (FBDG) (Flynn *et al.,* 2012). The number of servings of each food group consumed daily was calculated by adding the daily value of each relevant food item. The revised recommendations for the bread, cereal and potato group vary according to physical activity levels. For example, sedentary males and females aged ≥51 yrs are recommended to consume 4 and 3 servings per day, respectively while moderately active males and females are advised to consume 4-5 and 3-4 servings from this food group, respectively (Flynn *et al.,* 2012). Because information on physical activity levels were not recorded as part of the present study, males and females who consumed at least 4 and 3 servings, respectively were considered compliant with dietary recommendations for this food group.

3.5 Nutrient Intakes

A nutrient composition database was established for the ELDERMET FFQ using information compiled from the UK Food Standards Agency (FSA) nutrient databank (Food Standards Agency, 2002), with additional information from the Irish Food Consumption database (Black *et al.,* 2011) and manufacturers' information. The FSA food compositional databank was the primary nutrient data source used. For food items with several variations (e.g. different cuts of meat) prepared by different cooking methods, a range of appropriate foods were selected from the food compositional database and the average nutrient composition of that item calculated. Supplement intakes were included in the estimation of the nutrient intakes where appropriate.

The FSA nutrient databank (Food Standards Agency, 2002) provides two values for dietary fibre based on different measurement methods: the Englyst method (including non-starch polysaccharide (NSP) values) and the AOAC method (including lignin and resistant starch, in addition to NSP). Because values for a more comprehensive range of foods was available using the Englyst method, this dietary fibre value was used in our analysis, and intakes compared to the UK Department of Health recommendations for NSP (Department of Health, 1991).

Daily micronutrient intakes were compared to the dietary reference intakes, using the estimated average requirements (EAR) where available or adequate intakes (AI) for nutrients where no recommended daily allowance has been established (Flynn *et al.,* 2011; Institute of Medicine, 2011).

3.6 Micronutrient density

Variance in body size often determines gender-specific differences in energy and micronutrient intakes. For this reason, micronutrient intakes were adjusted for energy intakes. In line with other studies (Hannon *et al.,* 2001; O'Brien *et al.,* 2001), micronutrient density was calculated by dividing absolute nutrient intake by total energy intake (KJ) and amounts expressed per 10 MJ.

3.7 Statistical Methods

Statistical analyses were performed using PASW^{TM} version 18 statistical package. Statistical comparisons were made between males and females and between different age groups (64-74 yrs and ≥ 75 yrs). Differences between means were evaluated using

the Independent-samples t-test or Mann Whitney test, as appropriate. Differences between categorical distributions were evaluated using chi-square test or Fisher's exact test. A *P* value of <0.01 was considered as statistically significant.

4.0 Results

4.1 Demographic and Anthropometric Data

Selected baseline demographic and anthropometric characteristics are shown in Table 1. Mean age of study participants was 75 years. The majority of subjects had an adequate nutritional status (according to the MNA score ≥24). The mean BMI was 28.5 for males and 27.6 for females. The majority of subjects were classified as either overweight (45% of males, 34% of females) or obese (36% of males, 32% of females). A significantly larger proportion of females consumed dietary supplements compared to males (60% *vs.* 39%). The most commonly consumed supplements were minerals (23%), fish oil/fatty acids (23%), vitamins (16%) and multivitamin/multimineral (MVMM) preparations (11%). Over twice as many females used mineral supplements as males (32% *vs.* 13%), with calcium and calcium/vitamin D combination supplements being the most widely consumed supplement in this category.

4.2 Food consumption

Median daily food consumption data are presented in Table 2. Bread was consumed once to twice daily while wholegrain breakfast cereals and potatoes were consumed on average, once daily. Dairy foods, particularly milk and yoghurt were consumed once daily while eggs were consumed twice weekly. Two to three servings of fruit and approximately three servings of vegetables were consumed daily. Meat was consumed four to five times per week while fish and poultry were consumed one to two times per week. On average, four cups of hot beverages and two to three servings of desserts/sweets were consumed daily. Males consumed alcohol on average twice weekly while female consumption was limited to once per month. Food groups which were rarely/never consumed included pasta, rice, french fries, refined grain breakfast cereals, savoury snacks and soft drinks. Among the younger age category examined (64-74 yrs), females consumed significantly more wholegrain breakfast cereals, probiotics ($P<0.01$) and chicken/poultry ($P<0.001$), and significantly less meat, fish products, miscellaneous foods (sauces, ketchup, jams, preserves etc.), alcoholic drinks ($P<0.01$) and processed soups ($P<0.001$) than males of similar age. In the older age bracket (≥75 yrs), gender specific dietary choices were also apparent, with greater amounts of meat, chicken/poultry, potatoes and alcohol drinks being consumed by males compared with

females ($P<0.01$). Older participants (≥75 yrs) consumed significantly more desserts/sweets than the younger age group (64-74 yrs) for both genders ($P<0.01$).

4.3 Compliance with Dietary Recommendations

The Irish food-based dietary guidelines (FBDG) indicate the number of servings recommended for each of the four main food groups, in addition to guidelines for consumption of foods high in fat and/or sugar (Table 3). Daily food group consumption and compliance was measured with respect to recommendations for each food group. The median daily consumption of foods from the bread, cereal and potato food group was adequate (4.7 and 4.5 servings/day for males and females, respectively) with the majority of subjects (73.4% of males and 82.5% of females) compliant with the recommendations. Approximately half of subjects (47.9% of males and 57.9% of females) complied with recommendations to consume at least five servings of fruit and vegetables daily. Consumption of foods from the milk, cheese and yoghurt group was low among both genders with only 2.1% of males and 9.7% of females compliant with recommendations to consume 3 servings daily. On further analysis it was found that 26.6% of males and 25.4% of females consumed more than the recommended 3 servings daily, while 71.3% of males and 64.9% of females consumed less than the recommendation. Less than half of subjects (39.4% and 43.0%, males and females, respectively) consumed the recommended 2 servings of meat, fish and alternatives per day. Approximately 34.0% of males and 22.8% of females consumed more than the recommended amounts while 26.6% of males and 34.2% of females consumed less than the recommended 2 daily servings. Over half of those surveyed complied with guidelines to consume fish twice weekly (50% of males and 57% of females). Compliance with recommendations to consume foods high in fat and/or sugar sparingly (<3 servings/day) was low (10.6% and 19.3% males and females, respectively) with males consuming, on average 6.6 servings and females consuming 6.2 servings daily. Although no significant differences in compliance levels between gender and age groups were found, it is noteworthy that females from both age categories showed higher compliance rates than males with the dietary guidelines for all food groups.

4.4 Nutrient intakes

The median daily energy and macronutrient intakes from all sources and percentage energy contribution according to age group and gender are shown in Table 4. In general, elderly males had higher energy ($P<0.001$), protein and carbohydrates ($P<0.01$) intakes than females. Overall, the contribution to energy intakes from protein, carbohydrate and fat did not differ between males and females. Females had a significantly higher proportion of energy from total sugars than males (24.7% *vs.* 22.4%, $P<0.01$) while males consumed a significantly higher proportion of energy from alcohol (1.5% *vs.* 0.2%, $P<0.001$). Median daily alcohol intake for men was greater than that for women ($P<0.01$). Comparative analysis of energy and macronutrient intakes between genders aged 64-74 yrs showed no significant differences. Significant differences were found among the older age group (≥75 yrs) with males consuming significantly more energy, protein, carbohydrates ($P<0.001$) and fat ($P<0.01$) than females. Older males (≥75 y) had significantly higher energy and carbohydrate intakes ($P<0.01$) compared to younger males (64-74 yrs), with no such differences observed among females.

The percentage contribution of food groups to energy and macronutrient intakes were also investigated (data not shown). Food groups contributing the highest proportion to energy intakes were breads (16.7%), desserts/sweets (13.6%) and meat/poultry (10.2%). The main sources of protein were meat/poultry (26.4%), dairy foods (14.2%) and breads (14.2%) while fats/oils, including butter, spreads, salad dressings (19.7%), meat/poultry (15.7%) and desserts/sweets (13.4%) were the main contributors to fat intakes. The main dietary sources of carbohydrate were breads (24.1%), desserts/sweets (17.1%) and fruit (13.4%).

The median daily intake of vitamins and minerals from all sources, stratified by age and gender are shown in Table 5. In general, males had significantly higher intakes of thiamine, niacin, iron ($P<0.001$) and zinc ($P<0.01$) while females had significantly higher intakes of vitamin C ($P<0.01$). Older males (≥75 yrs) had significantly higher intakes of calcium and phosphorous ($P<0.01$) compared to those in the 64-74 age category. In contrast, older females (≥75 yrs) had significantly lower intakes of calcium and vitamin B6 than their younger counterparts ($P<0.01$).

The proportional contribution of food groups and nutritional supplements to key micronutrient (i.e. vitamin A, vitamin C, vitamin D, folate, calcium and iron) intakes were also investigated (data not shown). The contribution of dietary supplements to micronutrient intakes varied. Overall, the contribution of dietary supplements to micronutrient intakes was less than 9% with the exception of vitamin C and vitamin D. Fruit, vegetables and potatoes contributed 30.6%, 26.9% and 13.4%, respectively of the daily vitamin C intake while supplements contributed a further 10.1% to intakes. Dietary supplements were the main contributor to vitamin D intakes in this elderly group (28.5%), followed by fats/oils (12.2%) and fish (11.2%). Vegetables, soups and meat/poultry were the main contributors to vitamin A intakes (36.7%, 10.9% and 10.0% respectively), while the predominant contributors to folate intakes were vegetables (17.0%), breads (10.9%), potatoes (10.8%) and breakfast cereals (10.5%). The main dietary sources of iron were breads (22.1%), breakfast cereals (16.7%) and meat/poultry (11.3%), while the main sources of calcium were dairy foods (27.9%), breads (14.7%), breakfast cereals (13.5%) and dietary supplements (8.8%).

4.5 Micronutrient density

Daily intakes of vitamins and minerals were expressed per 10 MJ of energy and stratified by age and gender (Table 6). Dietary intake was generally more nutrient dense among females for most micronutrients and significantly for some including: vitamin A, vitamin B12, biotin, pantothenate, vitamin C, calcium, magnesium phosphorous and copper ($P<0.01$). Among older subjects (≥75 yrs), less disparity in nutrient density was found between genders with only a higher intake of vitamin C in females compared to males ($P<0.001$). This may be due to the fact that there was a significant reduction in micronutrient density between younger and older females for a number of micronutrients including several B vitamins, calcium and magnesium ($P<0.01$). There was no significant difference in micronutrient density between younger (64-74 yrs) and older (≥75 yrs) males.

4.6 Recommended Nutrient Intake Adequacy

The contribution of protein and carbohydrate to total energy intakes were within the target ranges (10-35% and 45-65%, respectively) (Flynn *et al.*, 2011). The percent of energy derived from total fat was at the upper limit of the target range (20-35%) (Flynn

et al., 2011), providing 34% and 35% of total energy for males and females, respectively, while the energy contribution from saturated fat was above the recommended target (<10%) (Flynn *et al.,* 2011), providing 14% and 15% of total energy for males and females, respectively. Non-starch polysaccharide (NSP) intakes were just below the UK recommendations (>18g/d) (Department of Health, 1991) for elderly females (16.4g/d), while elderly males consumed adequate NSP (18.0g/d). A NSP intake of 18g has been shown to equate to a dietary fibre intake of approximately 25g based on population food intakes in the North/South Ireland Food Consumption Survey (Galvin *et al.,* 2001).

The proportions of the surveyed Irish, community-dwelling elderly subjects with micronutrient intakes below the estimated average requirements (EAR) are shown in Table 7. In general, adequate intakes were reported for vitamin E, thiamine, riboflavin, niacin, vitamin B6, vitamin B12 intakes, while a large proportion of male (81.9%) and female subjects (71.9%) had sub-optimal vitamin D intakes. Over one third of subjects (36.2% of males and 42.1% of females) did not meet the requirements for folate and a significantly higher proportion of males had vitamin C intakes below the EAR, compared to females (27.7% *vs.* 7% $P<0.01$). Approximately one third of subjects did not meet the average requirement for calcium intake (39.4% males and 36.0% females) while a significantly larger proportion of elderly males had inadequate intakes for magnesium compared to females (61.7% vs. 32.5%; $P<0.001$).

5.0 Discussion

In this cross-sectional study of 208 community-dwelling elderly subjects, food consumption, nutrient intakes and nutritional status was assessed. In accordance with previous reports (Corish & Kennedy, 2003; Guigoz, 2006; Romero-Ortuno *et al.*, 2011), none of the study participants were classified as malnourished, and a low proportion of subjects had a BMI less than 18.5 kg/m^2, indicative of being underweight. In addition, and consistent with previous findings (Andreyeva *et al.,* 2007; Corish and Kennedy, 2003; Irish Universities Nutrition Alliance, 2011), a large proportion of the study participants were classified as overweight or obese. However, the definition of the optimal BMI among the elderly remains controversial as the relevance of a high BMI becomes less pronounced with ageing (Corrada *et al.,* 2006; de Hollander *et al.,* 2012). Epidemiological findings on the relationship between BMI and mortality suggest an

increased mortality risk for elderly with BMI values < 25 (Corrada *et al.,* 2006; Dey *et al.,* 2001; Landi *et al.,* 2000). It has also been shown that BMI classification of overweight in the elderly did not increase the risk of all-cause mortality, and obesity (BMI >30 kg/m^2) increased the risk only modestly (de Hollander *et al.,* 2012). Evidence also indicates that excessive weight in old age may serve a protective function against osteoporosis (Albala *et al.,* 1996; Barrera *et al.,* 2004) and it has been suggested that the World Health Organisation (WHO) cut-off of 25 kg/m^2 for overweight may not be appropriate for older persons (de Hollander *et al.,* 2012).

The primary objective of the ELDERMET study was to determine the gut microbiota profile of elderly population groups. Because of the population group involved, the use of weighed food intakes and food diaries was not appropriate. The dietary assessment method employed was not therefore designed to capture dietary information at the micronutrient level. However, FFQs have been shown to be an appropriate tool for conducting dietary assessment in elderly populations (Klipstein-Grobusch *et al.,* 1998) and use of the recently published UK portion size database, applicable for elderly, community-dwelling individuals (Wrieden & Barton, 2006) increased the accuracy of estimating nutrient intake in this population cohort. Some potential limitations of our findings must, however, be taken into account. FFQs inherently rely on a number of assumptions when estimating nutrient intakes; they contain broad food descriptions (e.g. lamb) rather than specific foods and cooking methods (e.g. grilled lamb chops) and therefore an average nutrient composition is assigned to each FFQ item based on the mean of a range of appropriate foods. Furthermore, in the present study, standard portion sizes for community-dwelling elderly subjects were used to determine daily nutrient intakes. Although these methods infer a number of assumptions, they are currently the most accurate and appropriate methods for this population group. The problem of assessing adequacy of micronutrient intakes at a population level is a long standing one (Carriquiry, 1999; Román-Viñas *et al.,* 2009) with comparison to Recommended Daily Allowance (RDA) values leading to an overestimation of nutrient inadequacy (Carriquiry, 1999). In this study, the prevalence of inadequate micronutrient intakes was estimated from the percentage of the population with median daily intake levels below the estimated average requirement (EAR; the average daily intake estimated to meet the requirement of fifty percent of healthy individuals in a particular age or gender group) as recommended (De Lauzon *et al.,* 2005). The present study

lacked information on physical activity, therefore comparisons to reference energy recommendations and estimations of underreporting were not performed for this population group. It is well acknowledged that dietary surveys are affected by varying degrees of underreporting (Goldberg & Black, 1998). However, the validity of methods to determine underreporting in elderly populations has not been thoroughly evaluated. As the prevalence of underreporting was not established in the present study cohort, results should be interpreted with caution. Finally, as the study population was self selected, it is possible that our study participants had better mental and physical health. Consequently, our study results cannot be generalised to the entire Irish community-dwelling elderly population.

Previous studies of food consumption report different eating patterns among older populations compared with their younger counterparts. In general, older persons traditionally consume three meals a day (Shahar *et al.,* 2003) with a decline in food quantity (Wakimoto & Block, 2001; Zhu *et al.,* 2010) and dietary variety (Fanelli and Stevenhagen, 1985; Kwon *et al.,* 2005) and an increase in dietary supplement usage with age (Irish Universities Nutrition Alliance, 2011; Radimer *et al.,* 2004). In line with other studies, approximately half of those surveyed in the present study consumed some form of dietary supplement (Bailey *et al.,* 2011; Irish Universities Nutrition Alliance, 2011; Johnson *et al.,* 2000; Schwarzpaul *et al.,* 2006).

Although a high level of fruit and vegetable consumption was evident in the cohort analysed, over one third of elderly subjects did not meet the recommended EAR for folate intake. Inadequate folate intakes, common amongst older populations (Elmadfa, 2009; Volkert *et al.,* 2004; Zhu *et al.,* 2010) are associated with increased plasma levels of homocysteine, an independent risk factor for heart disease and stroke (Zhu *et al.,* 2010). Additional supplementation of this important nutrient may be required to address nutritional inadequacy among elderly populations.

Despite relatively good compliance with guidelines for certain food groups, and in contrast to previous findings for elderly Irish subjects (Harrington *et al.,* 2008) the majority of subjects failed to meet the recommended guidelines for dairy foods with intakes among both genders. Avoidance of dairy products in older adults may be due to actual or perceived lactose intolerance (Lichtenstein *et al.,* 2008; Russell *et al.,* 1999) or misperceptions linking milk product consumption to weight gain or poor lipid profiles

(Eddy *et al.,* 1999; Horwath *et al.,* 1995). One third of participants in this study had calcium intakes below the EAR. In addition, vitamin D intakes were sub-optimal among both males and females with over two-thirds of subjects reporting intakes below the EAR. Not only is vitamin D vital for adequate intestinal calcium absorption (Cashman *et al.,* 2012), deficiency has been associated with several chronic conditions including cardiovascular disease, autoimmune disease, cancer (Souberbielle *et al.,* 2010) and mortality (Holick, 2007). However, inadequacy is widespread among elderly population groups (Elmadfa, 2009; Irish Universities Nutrition Alliance, 2011). The level of dietary supplementation reported in the present study is in line with estimates from the recent National Adult Nutrition Survey (Irish Universities Nutrition Alliance, 2011), contributing to over one quarter of dietary vitamin D intakes. Dietary supplements also played a significant contribution to calcium intakes (8.8%) in this population group. However, despite the high contribution of supplement use to both vitamin D and calcium intakes, the rationale for poor dietary intakes of such vital nutrients needs to be investigated and potentially more targeted, food-based strategies, such as food fortification of selected relevant foods are required to address this issue.

Excessive consumption of foods high in fat and/or sugar was prevalent among this elderly cohort and has been reported previously (Harrington *et al.,* 2008). Desserts/sweets were one of the main contributors to energy and fat in the elderly diet. High consumption of desserts/sweets and soft drinks is of particular concern as excessive intakes of high fat and/or sugar foods may contribute to the development of chronic health problems including type 2 diabetes and overweight/obesity. Energy dense foods tend to possess little nutritional value and frequent consumption of these foods often displace more nutrient-dense foods from the diet (Kant, 2003; van Dam & Seidell, 2007).

In line with findings from similar cohort studies (Elmadfa, 2009; Irish Universities Nutrition Alliance, 2011; Department of Health/Food Standards Agency, 2012), fat intakes were high in this elderly population group. In addition, intakes of saturated fat were excessive and may contribute to high BMI values, raised blood cholesterol and increase the risk of heart disease (Flynn *et al.,* 2011). Reports of high intakes of saturated fat are commonplace among Northern European population groups (Elmadfa, 2009; Pryer *et al.,* 2001b), while saturated fat intakes are lower among Southern

European elders (Moreiras *et al.,* 1991; Tur *et al.,* 2005), potentially due to high consumption of mono- and polyunsaturated fats.

Gender differences in food consumption among older populations have previously been reported. While females tend to have healthier dietary patterns than their male counterparts (Anderson *et al.,* 2011; Bamia *et al.,* 2005; Bates *et al.,* 1999; Prättälä *et al.,* 2007), males tend to consume more meat, potatoes, bread and alcohol and less fruits, vegetables, fish, chicken, cheese and sweets than women (Prättälä *et al.,* 2007). In general, females tend to consume more dietary supplements than males (Bailey *et al.,* 2011; Irish Universities Nutrition Alliance, 2011; Johnson *et al.,* 2000; Schwarzpaul *et al.,* 2006) and female food behaviours tends to be more in accordance with the dietary guidelines (Roos *et al.,* 1998). In the current study, use of dietary supplements was more common among females than males. In addition, the observed gender differences in food group intakes are consistent with those previously reported (Arganini *et al.,* 2012; Bates *et al.,* 1999). It has been shown that when making food choices, males rank health as a lower priority to other considerations such as taste and convenience (Donkin *et al.,* 1998; Steptoe *et al.,* 2002; Wardle & Griffith, 2001). Older males (55-64 yrs) also tend to have poorer knowledge about current dietary recommendations with fewer men aware of the links between diet, health and disease (Baker and Wardle, 2003). Furthermore, gender differences in food consumption may be related to social norms and cultural beliefs (Prättälä *et al.,* 2007) with elderly women often maintaining the traditional role of choosing, preparing and cooking food (Arganini *et al.,* 2012; Donkin *et al.,* 1998; Prättälä *et al.,* 2007).

Dietary intakes of elderly females were more nutrient-dense than those of elderly males, as shown previously (Pryer *et al.,* 2001b; Volkert *et al.,* 2004). In addition, the prevalence of inadequate micronutrient intakes was higher among males than females and may reflect the observed differences in food consumption and dietary supplements usage. A higher proportion of elderly males consumed inadequate amounts of vitamin C compared to elderly females, a trend evident in both younger and older age categories. Higher vitamin C intakes have previously been reported in the UK among elderly females compared to males (65-75 yrs) and have been attributed to increased consumption of ‘vitamin C-rich’ foods such as fruit and vegetables (Bates *et al.,* 1999). Although not significantly different, females consumed more fruit than elderly males in

the current study. Age-related decreases in antioxidant enzyme activity, reported to contribute to increases oxidative damage and age related degeneration (Chernoff, 2005; Elmadfa & Meyer, 2008) highlight the requirement for a continuous supply of key antioxidant nutrients, including vitamin C. The occurrence of inadequate zinc and magnesium intakes were particularly high among males and may have negative health implications in this group. Therefore, older adults, particularly elderly men, should be encouraged to eat rich dietary sources, such as shellfish, legumes, nuts/seeds and whole-grain cereals.

For the majority of food groups studies there was no significant difference in intakes between the younger (64-74 yrs) and older (≥75 yrs) elderly participants, with the exception of desserts/sweets where there was a marked increase in consumption with age. Similarly, a study examining dietary intakes over a ten year follow-up in a group of elderly Italians found a significant increase in sweet-eating with age (Toffanello *et al.*, 2010). Altered olfactory function including a reduced sense of taste associated with ageing may be responsible for this preference for softer, sweeter, more palatable foods (Toffanello *et al.*, 2010). Indeed, elderly women with olfactory dysfunction have a higher intake of sweets than those without dysfunction (Duffy *et al.*, 1995). This increased consumption of desserts/sweets with age may displace more nutrient-dense foods from the diet and increase the risk of obesity-induced chronic disease disorders, including type 2 diabetes. In the present study, the female diet appeared to deteriorate with advancing age: older females (≥75 yrs) had a lower micronutrient-dense diet than younger females (64-74 yrs).

To conclude, this study highlights the main foods contributing to nutrient intakes among older Irish persons and points to several inadequacies among this vulnerable population group. Our results highlight the need to improve awareness among elderly populations regarding the importance of nutritious dietary choices for long-term health. Interventions to improve dairy consumption, and reduce dietary fat (particularly saturated fat) intakes among elderly population groups may also be warranted. In addition, specific micronutrient supplementation may be necessary, particularly among elderly males, to ensure adequate micronutrient intakes. Dietary recommendations specific to the nutritional needs and health concerns associated with ageing are required as improvement of nutritional status may lead to reduced ill-health, help maintain a

better quality of life and promote longer independent living (Harris & Haboubi, 2005). In addition, the association between diet and the intestinal microbiota outlined in a previous study by the ELDERMET project (Claesson *et al.*, 2012) highlights the importance of promoting healthy ageing by nutritional interventions which target the gut microbiota. Our findings present both challenges and opportunities to the food industry and healthcare policy makers: to identify dietary vehicles suitable for fortification and to devise age-specific, dietary recommendations to address the nutritional concerns of elderly males and females.

Table 1. Selected descriptive characteristics of Irish community-dwelling elderly subjects (*n* 208) stratified by age group and gender

Characteristics[a]	All ages				64-74 years				≥75 years			
	Males (*n* 94)		Females (*n* 114)		Males (*n* 45)		Females (*n* 57)		Males (*n* 49)		Females (*n* 57)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Age *(yrs)*	75.4	6.5	74.8	6.7	69.9††	2.6	69.1††	2.9	80.4	4.7	80.5	3.9
Smoking status *(%)*												
Never	42		52		36		55		47		48	
Former smoker	53		42		54		43		53		41	
Current	5		6		10		2		0		11	
Weight *(kg)*	83.2	14.3	69.3**	15.1	87.5	15.6	70.8**	14.6	79.4†	11.9	67.9**	15.6
Height *(cm)*	170.4	7.2	158.5**	5.1	172.3	7.2	159.0**	5.9	168.7†	6.7	158.1**	4.2
BMI[b] (kg/m^2)	28.5	4.1	27.6	6.1	29.2	4.2	28.1	5.9	27.9	4.0	27.1	6.4
BMI, kg/m^2 categories *(%)*												
Underweight (<18.5)	1		2		0		0		2		4	
Normal (18.5-24.9)	18		32		13		32		22		32	
Overweight (25-29.9)	45		34		44		36		45		32	
Obese (≥30)	36		32		42		32		31		32	
MNA[c] score *(0, 30)*	26.0	2.0	25.8	2.2	25.9	2.0	26.2	2.0	26.1	2.1	25.4	2.3
Nutritional status *(%)*												
Normal Nutritional Status (MNA ≥24)	90		87		91		11		90		84	
At risk of malnutrition (MNA 17-23.5)	10		13		9		89		10		16	
Malnourished (MNA<17)	0		0		0		0		0		0	
Dietary Supplement Users (%)	39*		60		31**		68		47		51	
MVMM[d] Supplements (%)	6		15		9		21		4		9	
Vitamin Supplement (%)	17		15		16		18		8		12	
Mineral Supplements (%)	13*		32		9**		40		16		23	
Fish Oil/Fatty acids (%)	18		27		22		33		14		21	
Amino acids (%)	3		3		4		5		2		0	
Glucosamine (%)	3		10		4		14		2		5	
Food Supplement (%)	1		3		0		0		2		5	
Herbal Supplements (%)	4		8		7		14		2		2	

[a]Values represent mean and standard deviation (SD) or percentages (%)

[b]Body Mass Index [c]Mini-Nutritional Assessment: Score out of a total of 30. Higher scores indicate a higher degree of nourishment

[d]Multivitamin/Multimineral supplements

*P≤ 0.01; **P ≤ 0.001: comparisons between males and females (Independent-samples t test or chi-square test).

†P≤ 0.01; ††P≤0.001: comparisons between age groups within each gender category (Independent-samples t test)

Table 2. Median daily intake (g/d) of food groups among Irish community-dwelling elderly subjects stratified by age group and gender

	All ages				64-74 years				≥75 years			
	Males (*n* 94)		Females (*n* 114)		Males (*n* 45)		Females (*n* 57)		Males (*n* 49)		Females (*n* 57)	
Food groups (g/d)	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
Brown breads	78	27-117	58	19-103	66	25-135	62	19-106	107	23-113	54	17-99
White breads	33	6-107	14*	2-50	30	3-109	8	2-49	36	7-108	14	2-53
Wholegrain breakfast cereals	53	4-225	161	31-219	42*	0-200	185	29-219	87	30-225	122††	25-161
Refined grain breakfast cereals	0	0-1	0	0-0	0	0-1	0	0-0	0	0-0	0	0-0
Pasta/rice	2	0-17	4	0-24	2	0-18	4	0-30	2	0-17	4	0-18
Potatoes	153	98-172	131**	80-146	144	97-173	140	81-148	156	120-168	127**	73-137
French fries	4	1-18	3*	1-16	4	1-18	3	1-16	15	1-17	3	1-15
Fruit	178	96-313	223	153-349	184	112-276	246	125-384	171	82-379	223	159-315
Vegetables	150	109-222	155	117-236	151	108-229	156	118-273	145	110-215	153	105-226
High fat dairy	30	5-129	63	9-135	25	8-128	57	4-150	66	4-129	69	14-133
Low fat dairy	1	0-119	4	0-101	1	0-85	9	0-118	1	0-188	0	0-96
Meat	52	39-73	41**	22-56	51	34-68	36*	17-56	58	39-76	47*	30-56
Meat products	11	2-22	7*	2-12	10	2-22	8	2-11	12	2-23	7	2-15
Fish	19	7-31	20	6-38	19	5-31	29	12-41	19	12-30	19	6-34
Fish products	1	0-17	0*	0-6	1	0-18	0*	0-3	1	0-17	1	0-15
Poultry	12	12-24	12	11-26	12**	12-25	26	12-26	12	12-24	11*††	11-23
Poultry products	0	0-1	0	0-0	0	0-4	0	0-0	0	0-1	0	0-0
Eggs	21	10-52	20	11-22	24	9-59	20	13-22	21	10-52	20	10-23
Salad dressings	0	0-4	2	0-4	0	0-4	3	0-4	0	0-4	2	0-4
Butter	0	0-20	1	0-20	2	0-20	0	0-18	0	0-20	1	0-20
Spreads	10	0-22	7	0-20	8	0-22	9	0-18	20	0-20	2	0-20
Fresh soups	8	0-79	30	0-67	6	0-64	35	2-67	9	0-95	6	0-63
Processed soups	6	0-35	0	0-39	6	0-33	0**†	0-4	4	0-60	4	0-62
Desserts/sweets	77	39-135	61	37-101	59†	24-108	50†	25-83	97	52-157	80	46-110
Savoury snacks	0	0-1	0	0-1	0	0-1	0	0-1	0	0-0	0	0-0
Soft drinks	1	0-39	0	0-45	3	0-41	0	0-10	1	0-39	3	0-47
Hot beverages	776	557-1070	764	559-1018	776	394-1079	849	575-1139	760	579-1094	679	485-1017
Alcoholic drinks	38	1-126	4**	0-37	83	3-160	15*	1-45	18	0-115	1*††	0-17
Probiotics	0*	0-29	9	0-100	0*	0-50	29	0-100	0	0-22	0	0-100
Miscellaneous	31	17-58	22**	9-36	26	17-60	17*	7-33	34	20-58	26	11-43

Values represent median and interquartile ranges (IQR)

*P≤ 0.01; **P≤0.001: comparisons between males and females within each age category and both age categories combined (Mann-Whitney test)

†P≤ 0.01; ††P≤0.001: comparisons between age groups within each gender category (Mann-Whitney test)

Table 3. Median daily intake (servings/d) of food groups among Irish community-dwelling elderly subjects compared to recommended dietary guidelines for elderly individuals stratified by age group and gender

Food groups (servings/d)	Recommended	All ages				64-74 years				≥75 years			
		Males (*n* 94)		**Females** (*n* 114)		**Males** (*n* 45)		**Females** (*n* 57)		**Males** (*n* 49)		**Females** (*n* 57)	
		Median	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
Bread, Cereals & Potatoes	**4+ (3+)**	4.7	3.8 -6.3	4.5	3.3-5.5	4.4	3.4-5.3	4.2	3.2-5.3	5.0	4.2-6.6	4.6	3.4-5.7
Fruit & Vegetables	**5+**	4.9	3.2-7.1	5.8	4.1-8.5	4.9	3-2-7.1	6.4	4.2-8.7	4.8	3.1-7.4	5.4	3.9-7.4
Milk, Yogurt and Cheese	**3**	1.4	0.7-4.2	1.4	0.7-3.6	1.3	0.7-2.3	1.4	0.7-4.1	1.6	1.0-5.0	1.3	0.7-3.5
Meat, Fish and Alternatives	**2**	2.1	1.6-2.8	1.8	1.5-2.3	1.9	1.2-2.5	1.7	1.5-2.2	2.1	1.7-2.8	1.9	1.5-2.4
Foods high in fat and/or sugar	**Sparingly <3**	6.6	4.4-9.4	6.2	3.8-8.6	6.2	3.6-9.0	5.2	2.9-7.9	6.9	5.0-10.8	6.5	4.0-8.6

Values in parentheses for females

Values represent medians and interquartile ranges (IQR)

Table 4. Median daily intakes of macronutrients and alcohol from all sources (including dietary supplements) among Irish community-dwelling elderly subjects stratified by age group and gender

	All ages				64-74 years				≥75 years			
	Males (*n* 94)		Females (*n* 114)		Males (*n* 45)		Females (*n* 57)		Males (*n* 49)		Females (*n* 57)	
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Energy (kJ)	8440	6981-9725	6991**	5859-8725	7932†	6646-9196	7144	5446-9009	9231	7639-10024	6988**	6023-8508
Energy (kcal)	2008	1661-2315	1668**	1396-2068	1883†	1580-2187	1701	1288-2139	2194	1815-2389	1666**	1433-2025
Protein (g)	79.2	67.8-92.7	67.7**	57.6-85.3	77.5	61.5-86.1	67.3	58.6-89.1	83.9	74.6-97.2	68.1**	54.5-80.9
Fat (g)	77.2	59.4-94.3	64.0	49.4-88.9	70.6	54.8-91.2	63.9	46.5-94.5	80.8	65.1-98.8	64.0*	51.5-81.9
Saturated fat (g)	30.3	24.4-39.1	25.6	20.2-40.0	27.5	21.9-35.2	25.8	19.1-42.5	33.9	25.6-40.4	25.6*	21.1-38.0
Carbohydrate (g)	246.4	203.9-293.7	203.6*	174.2-277.0	233.0†	190.8-264.4	200.4	162.7-282.3	265.5	224.1-323.8	211.3**	182.1-263.7
Total sugar (g)	104.6	80.9-143.7	104.7	77.2-137.5	96.1	73.3-121.3	105.3	77.0-144.0	113.2	89.7-162.5	101.6	75.9-131.7
NSP (g)	18.0	13.7-22.6	16.4	12.9-20.4	16.7	12.8-20.2	16.6	14.1-21.2	19.6	14.4-24.0	15.8	11.9-20.4
Alcohol (g)	3.7	0.2-11.0	0.4**	0.1-3.4	4.4	0.4-13.0	1.6*	0.1-4.2	1.7	0.2-10.8	0.2*	0.1-2.6
Protein (%TE)	16.0	14.4-17.7	16.2	14.3-18.2	16.0	14.3-18.5	16.9	14.2-18.5	15.7	14.5-17.4	15.7	14.2-17.7
Fat (%TE)	34.0	31.6-38.1	35.1	31.8-38.9	33.9	31.3-38.2	35.0	31.3-38.8	34.7	31.8-37.7	35.4	32.5-39.0
Saturated fat (%TE)	14.0	11.9-16.0	15.0	12.3-17.4	12.9	11.9-16.1	14.9	12.0-17.9	14.4	11.6-16.1	15.1	12.5-17.0
Carbohydrate (%TE)	49.9	45.8-53.9	50.1	47.2-54.3	49.7	45.0-53.6	49.5	47.0-54.3	50.4	46.9-54.5	50.4	47.8-54.3
Total sugar (%TE)	22.4*	17.2-26.0	24.7	21.6-27.9	21.4*	16.8-25.5	24.7	21.5-28.8	23.5	18.0-26.4	24.7	21.7-27.3
Alcohol (%TE)	1.5	0.1-4.0	0.2**	0.0-1.6	2.1	0.1-4.9	0.6	0.1-1.9	0.5	0.0-3.8	0.1	0.0-1.0

NSP, Non-starch polysaccharide (Englyst method); TE, Total Energy

Values represent medians and interquartile ranges (IQR)

*P≤ 0.01; **P≤0.001: comparisons between males and females within each age category and both age categories combined (Mann-Whitney test)

†P≤ 0.01; ††P≤0.001: comparisons between age groups within each gender category (Mann-Whitney test)

Table 5. Median daily intakes of micronutrients from all sources (including dietary supplements) among Irish community-dwelling elderly subjects stratified by age group and gender

	Target	All ages				64-74 years				≥75 years			
		Males (*n* 94)		Females (*n* 114)		Males (*n* 45)		Females (*n* 57)		Males (*n* 49)		Females (*n* 57)	
		Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Vitamins:													
Vitamin A (µg RE)[a]	625 (500)	1208.5	801.8-1739.2	1308.6	807.2-1958.7	1105.5	788.0-1645.6	1302.8	802.2-1893.7	1324.8	936.3-1839.0	1327.6	759.5-1971.6
Vitamin D (µg)[a]	10	5.1	2.8-7.3	4.8	3.0-11.4	5.0	2.8-7.1	5.5	3.4-13.1	5.3	2.8-8.4	4.0	2.6-10.2
Vitamin E (mg)[a]	12	29.7	21.8-36.6	28.4	20.9-35.9	29.5	20.5-38.0	29.1	23.0-39.5	30.8	22.2-36.3	26.1	19.7-33.7
Thiamin (mg)[a]	1.0 (0.9)	1.9	1.6-2.4	1.7**	1.3-2.2	1.9	1.6-2.2	1.7	1.4-2.3	2.0	1.7-2.5	1.6**	1.2-2.0
Riboflavin (mg)[a]	1.1 (0.9)	2.0	1.5-2.8	1.8	1.4-2.9	2.0	1.3-2.4	2.2	1.6-3.2	2.3	1.7-3.1	1.7*	1.4-2.7
Niacin (mg NE)[a]	12 (11)	36.4	30.7-42.9	30.7**	25.0-39.6	33.9	30.3-41.7	33.4	25.3-40.7	36.8	31.0-44.3	30.6**	23.4-35.4
Vitamin B6 (mg)[a]	1.4 (1.3)	2.7	2.2-3.5	2.6	2.0-3.4	2.6	2.2-3.5	3.0	2.1-4.1	2.7	2.1-3.4	2.4†	1.8-3.0
Vitamin B12 (µg)[a]	2.0	6.9	4.5-9.1	6.7	4.7-9.6	6.4	3.8-8.5	7.8	5.2-10.7	7.1	4.6-9.5	6.0	4.0-8.8
Folate (µg)[a]	320	375.2	282.5-527.9	358.0	266.7-485.8	363.8	273.8-521.2	406.9	297.0-540.5	377.3	290.5-538.0	324.1	253.5-438.6
Biotin (µg)[b]	30	49.3	40.0-63.2	45.3	35.0-65.3	47.4	39.0-58.7	47.2	37.9-82.4	50.6	40.5-64.3	44.3	30.2-56.7
Pantothenate (mg)[b]	5	7.2	5.8-9.8	6.7	5.2-9.2	7.0	5.2-8.3	7.3	5.8-10.1	7.3	6.1-10.2	6.3*	5.1-8.6
Vitamin C (mg)[a]	75 (60)	95.5*	72.1-139.7	115.2	84.3-175.0	92.5	70.5-135.5	129.2	87.2-188.6	98.0	72.5-141.5	108.7	84.0-154.7
Minerals:													
Calcium (mg)[a]	800/1000[d] (1000)	1056.2	829.7-1437.0	1272.2	796.7-1640.7	900.4**†	704.7-1296.9	1415.7	922.5-1747.3	1193.5	945.5-1582.5	1020.9†	774.9-1440.5
Magnesium (mg)[a]	350 (265)	318.7	267.7-394.5	305.3	251.6-397.8	307.6	255.6-373.8	307.7	255.4-428.8	331.2	280.1-419.0	297.8	223.6-353.6
Phosphorous (mg)[a]	580	1461.7	1231.2-1738.2	1336.1	1051.3-1732.5	1365.7†	1120.9-1683.6	1331.0	1099.9-1786.6	1523.7	1311.2-1803.5	1338.5*	1021.7-1627.2
Iron (mg)[c]	7 (6)	13.3	11.7-15.5	11.1**	8.8-14.6	12.6	11.0-15.2	11.6	9.3-15.2	13.9	12.2-16.2	10.9**	8.7-14.3
Copper (mg)[a]	0.7	1.3	1.1-1.5	1.1	0.9-1.6	1.2	1.0-1.4	1.2	1.0-1.6	1.3	1.1-1.6	1.1	0.9-1.6
Zinc (mg)[a]	9.4 (6.8)	10.5	9.1-13.1	8.9*	7.6-11.9	10.4	7.9-12.8	9.7	7.9-12.3	10.6	9.4-13.4	8.6**	7.5-11.4

RE, retinol equivalents; NE, Niacin Equivalents; values in parentheses are for women. [a] Estimated Average Requirement (EAR) from Institute of Medicine (Institute of Medicine, 2011); [b] Adequate Intake (AI) from Institute of Medicine (Institute of Medicine, 2011)

[c] Estimated Average Requirement (EAR) from Food Safety Authority of Ireland (Flynn *et al.*, 2011); [d] 800 mg (males 51-70 y); 1000 mg (males >70 y). Values represent medians and interquartile ranges (IQR)

*P≤ 0.01; **P≤0.001: comparisons between males and females within each age category and both age categories combined (Mann-Whitney test)

†P≤ 0.01; ††P≤0.001: comparisons between age groups within each gender category (Mann-Whitney test)

Table 6. Median daily intakes of micronutrients per 10 MJ total energy among Irish community-dwelling elderly subjects stratified by age group and gender

	All ages				64-74 years				≥ 75 years			
	Males (*n* 94)		Females (*n* 114)		Males (*n* 45)		Females (*n* 57)		Males (*n* 49)		Females (*n* 57)	
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Vitamins:												
Vitamin A (µg RE)	1416.7*	1071.9-2110.2	1700.9	1217.6-2448.5	1425.4	1094.7-2197.7	1659	1207.8-2526.4	1409.2	998.9-2020.9	1793.7	1259.5-2362.4
Vitamin D (µg)	5.4	3.6-8.7	6.4	3.9-14.7	5.7	3.7-8.5	6.6	5.2-17.6	5.0	3.6-9.8	5.7	3.7-11.7
Vitamin E (mg)	33.8	26.3-44.1	37.6	28.8-48.0	38.9	26.1-47.2	39.9	31.2-54.2	33.3	26.2-38.7	35.4	24.3-45.7
Thiamin (mg)	2.3	2.0-2.7	2.3	2.0-2.7	2.2	2.0-2.7	2.5	2.1-2.9	2.3	2.0-2.6	2.2†	2.0-2.6
Riboflavin (mg)	2.4	2.0-3.0	2.7	2.2-3.4	2.3*	2.0-2.7	2.9	2.4-4.0	2.5	2.1-3.2	2.5†	2.1-3.2
Niacin (mg NE)	42.5	37.9-48.8	43.4	37.4-49.4	44.0	39.0-51.3	44.3	40.7-51.5	42.1	37.2-47.1	41.1	36.4-46.5
Vitamin B6 (mg)	3.3	2.5-4.2	3.6	2.9-4.7	3.5	2.8-4.4	4.2	3.4-5.3	3.1	2.3-3.7	3.1††	2.6-4.1
Vitamin B12 (µg)	7.9*	5.9-9.8	9.3	6.9-12.1	8.3*	5.9-10.0	10.8	8.0-13.6	7.8	5.9-9.6	8.2†	6.2-11.2
Folate (µg)	430.7	357.9-636.8	480.3	372.7-680.2	452.6	361.6-672.9	560.3	446.0-740.0	408.5	352.3-607.1	413.0†	358.2-588.7
Biotin (µg)	58.1*	49.7-67.2	62.8	54.7-75.8	57.5*	51.6-67.9	70	57.7-89.4	58.5	48.0-66.9	59.9††	52.1-67.2
Pantothenate (mg)	8.8*	7.5-10.0	9.6	8.0-11.4	8.8**	7.4-10.0	10.4	8.9-12.5	8.8	7.3-10.1	8.6†	7.7-10.2
Vitamin C (mg)	116.4**	82.5-164.6	157.5	123.5-244.4	126.7*	82.0-187.3	160.9	121.5-274.4	110.2**	77.8-157.7	151.9	125.1-193.0
Minerals:												
Calcium (mg)	1241.4**	994.8-1731.5	1580.8	1153.0-2075.4	1142.4**	943.7-1576.8	1831.1	1308.7-2260.3	1325.5	1110.1-1859.2	1359.6†	1064.0-1770.1
Magnesium (mg)	383.1*	342.7-454.2	426.1	355.0-489.3	373.1*	348.9-458.9	455.6	376.8-516.7	391.5	323.7-451.4	400†	353.1-459.7
Phosphorous (mg)	1728.1*	1537.7-1960.2	1883.2	1638.0-2108.9	1724.2**	1518.2-1957.0	1982.4	1736.5-2216.9	1759.4	1562.5-1969.2	1764.7	1601.7-2063.5
Iron (mg)	15.7	14.0-17.8	15.6	13.7-18.4	15.7	14.1-17.3	16.6	14.4-18.5	15.6	14.0-18.5	15.2	13.2-18.1
Copper (mg)	14.5**	13.4-16.6	16.3	14.2-19.0	14.4*	13.5-15.9	17	14.6-19.3	14.6	12.7-17.0	15.6	13.8-18.5
Zinc (mg)	12.8	11.1-14.2	12.6	11.3-14.9	13.0	11.3-14.9	13.2	11.8-15.4	12.3	11.1-13.8	12.3	11.0-14.1

RE, retinol equivalents; NE, Niacin Equivalents

Values represent medians and interquartile ranges (IQR)

*P≤ 0.01; **P≤0.001: comparisons between males and females within each age category and both age categories combined (Mann-Whitney test)

†P≤ 0.01; ††P≤0.001: comparisons between age groups within each gender category (Mann-Whitney test)

Table 7. Percentage of Irish community-dwelling elderly subjects with daily micronutrient intakes below the Estimated Average Requirements (EAR), stratified by age group and gender

		All ages		64-74 years		≥ 75 years	
	Target	**Males** (*n* 94)	**Females** (*n* 114)	**Males** (*n* 45)	**Females** (*n* 57)	**Males** (*n* 49)	**Females** (*n* 57)
Vitamins:							
Vitamin A (RE)[a]	625 (500) µg	13.8	5.3	11.1	1.8	16.3	8.8
Vitamin D [a]	10 µg	81.9	71.9	86.7	70.2	77.6	73.7
Vitamin E [a]	12 mg	1.1	5.3	2.2	3.5	0	7.0
Thiamin [a]	1.0 (0.9) mg	2.1	0.9	2.2	0	2.0	1.8
Riboflavin [a]	1.1 (0.9) mg	5.3	3.5	8.9	1.8	2.0	5.3
Niacin (NE)[a]	12 (11) mg	0	0	0	0	0	0
Vitamin B6 [a]	1.4 (1.3) mg	1.1	3.5	2.2	0	0	7.0
Vitamin B12 [a]	2.0 µg	1.1	1.8	2.2	0	0	3.5
Folate [a]	320 µg	36.2	42.1	40.0	35.1	32.7	49.1
Vitamin C [a]	75 (60) mg	27.7	7**	28.9	7*	26.5	7.0*
Minerals:							
Calcium [a]	800/1000[c] (1000) mg	39.4	36	48.9	26.3	30.6	45.6
Magnesium [a]	350 (265) mg	61.7	32.5**	66.7	28.1**	57.1	36.8
Phosphorous [a]	580 mg	0	1.8	0	0	0	3.5
Iron [b]	7 (6) mg	1.1	2.6	2.2	3.5	0	1.8
Copper [a]	0.7 mg	0	0	0	0	0	0
Zinc [a]	9.4 (6.8) mg	31.9	14.9*	37.8	8.8**	26.5	21.1

Values in parentheses are for women; RE, retinol equivalents

[a] Estimated Average Requirement (EAR) from Institute of Medicine (Institute of Medicine, 2011)

[b] Estimated Average Requirement (EAR) from Food Safety Authority of Ireland (Flynn *et al.*, 2011)

[c] 800 mg (males 51-70 y); 1000 mg (males >70 y)

*P ≤ 0.01; **P ≤ 0.001: comparisons between males and females within each age category and both age categories combined (Fisher's exact test)

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CHAPTER 4

Cognitive decline in non-diabetic elderly subjects associated with high glycaemic diet

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1.0 Abstract

The proportion of elderly people and the average lifespan are both increasing worldwide. Ageing is associated with loss of cognitive function and an increased risk of dementia which is expected to place growing demands on health and long-term care providers. Among multiple causative factors, evidence suggests that cognitive impairment in older subjects may be influenced by diet. The objective of this study was to examine the association between dietary patterns, dietary glycaemic index (GI) and glycaemic load (GL), and cognition in older Irish adults. Two hundred and eight community-dwelling subjects (94 males and 114 females; aged 64-93 years) were analysed. Dietary intake was assessed using a validated semi-quantitative food frequency questionnaire (FFQ). Cognitive capacity was tested using the Mini-Mental State Examination (MMSE). Hierarchical clustering was conducted on FFQs to derive patterns of dietary intake. Regression models were used to examine the relationship between dietary GI and GL, and MMSE score and adjusted for confounding variables. *Results:* Elderly subjects consuming 'prudent' dietary patterns (high in fruit, vegetables, fish, low-fat dairy, salad dressings and low in red meat and white bread) had higher MMSE scores than those consuming 'Western' dietary patterns (high in red meat, white bread and low in fruit and vegetables; $P<0.05$). Logistic and Poisson regression analyses both indicated that MMSE score was inversely associated with the GI and the GL of the diet ($P<0.05$) even after adjusting for age, gender, diabetes, hypertension, healthy food diversity and nutritional status. In conclusion, consumption of a high glycaemic diet is associated with poorer cognitive performance in this community-dwelling elderly Irish cohort.

2.0 Introduction

Recent decades have witnessed a dramatic increase in the life expectancy of elderly people worldwide (World Health Organisation/National Institute of Health, 2011). However, the number of years an individual can expect to live a healthy life has not kept pace (European Joint Action on Healthy Life Years, 2012). This has resulted in an increasing proportion of people living with age-related disabilities such as sarcopenia, depression and cognitive decline, placing growing demands on health and long-term care providers (World Health Organisation/National Institute of Health, 2011). In 2010, there were an estimated 35.6 million people over 60 years of age with dementia worldwide and estimates suggest that these figures will almost double every 20 years to 65.7 million by 2030 and 115.4 million by 2050 (Prince *et al.*, 2013).

Cognitive impairment can lead to significant loss of functional independence and plays a major role in overall age-related deterioration (McGuire *et al.*, 2006). Therefore maintenance of cognitive function with aging is a current and growing concern. It has been proposed that diet may play a fundamental role in slowing the progression of age-related decline (Allès *et al.*, 2012; Parrott & Greenwood, 2007). Traditionally, studies have investigated the effect of single nutrients or foods (e.g. fish, polyunsaturated fatty acids, antioxidant micronutrients, and folate) on cognition (Smith & Blumenthal, 2010). More recently, much attention has focussed on the associations between dietary patterns (which represent a combination of foods) and cognition (Allès *et al.*, 2012).

Dietary patterns are increasingly used in current nutrition epidemiology. This approach is mainly justified by the fact that theoretically, people do not eat isolated nutrients, but rather meals composed of a variety of foods, with complex combinations of nutrients which may interact (Villegas *et al.*, 2004). There has been a relatively rapid global shift in dietary patterns from diets high in complex carbohydrates and fibre, to what has been labelled the 'Western diet', characterised by high proportion of fat and refined sugars, with reduced fruit, vegetable and fibre consumption (Francis & Stevenson, 2013). High saturated fat and refined sugar intakes, typical of the modern Western diet, have been associated with a number of adverse health conditions, most notably obesity and type 2 diabetes (T2D) (Kanoski & Davidson, 2011; Steyn *et al.*, 2004; van Dam *et al.*, 2002). It has also been proposed that the Western diet may be associated with cognitive impairment (Francis & Stevenson, 2013; Kanoski & Davidson, 2011; Torres *et al.*,

2012), while a number of studies have shown that healthful dietary patterns characterised by high consumption of wholegrain cereals, fruit, vegetables and fresh fish may help attenuate age-related cognitive decline in older populations (Akbaraly *et al.*, 2009a; Wengreen *et al.*, 2009).

Recently, much interest has focussed on the effect of dietary glycaemic index (GI) and glycaemic load (GL) on health (Brand-Miller *et al.*, 2009). The GI is a measure of the quality of dietary carbohydrate and how quickly it is absorbed into the bloodstream following consumption. Carbohydrates with a high GI are associated with greater fluctuations in blood glucose and insulin concentrations than those with a low GI (Foster-Powell *et al.*, 2002). GL reflects both the quality and quantity of the carbohydrate and provides a summary measure of the relative glycaemic impact of a typical serving of a particular food. Foods with a high GL elicit larger glycaemic and insulinemic responses (Barclay *et al.*, 2005).

High-GI/GL diets have been proposed to contribute to insulin resistance and diabetes development by rapidly increasing postprandial glucose and insulin concentrations (Dong *et al.*, 2011; Sluijs *et al.*, 2013). In addition, diabetes and insulin resistance have been associated with an increased risk of cognitive impairment (Kalmijn *et al.*, 1995; Reijmer *et al.*, 2010; Watson & Craft, 2010). However, there is a paucity of research investigating the long-term effect of high GI/GL diets on cognitive function in humans. It has been shown that in the short-term, consumption of high GI meals resulted in postprandial memory impairment in healthy adults (Nabb & Benton, 2006) and diabetics (Greenwood *et al.*, 2003; Papanikolaou *et al.*, 2006). Conversely, Luchsinger and colleagues found no relationship between calorie-adjusted GL and the risk of Alzheimer's disease in a cohort of elderly subjects (Luchsinger *et al.*, 2007).

Mounting evidence indicates a role for nutrition-related factors in lowered loss of function decline during brain aging. It is still unknown what, if any, relationships exist between dietary GI/GL in the elderly and cognitive function. The present study was based on the hypothesis that cognitive decline in non-diabetic elderly subjects is associated with a Western dietary pattern and a high glycaemic diet. The objective of this study was therefore to examine the association of dietary patterns, dietary GI and GL with cognition in an elderly population group.

3.0 Materials and Methods

3.1 Study Participants

The study population comprised a sub-set of self-selected, community-dwelling ELDERMET project participants (*n* 208; 94 males, 114 females; 64-93 years), who responded to advertising in local health-care and general practice centres, active retirement groups and events, information sessions and local media from the Cork city and county region of southern Ireland. Community-dwelling, day-hospital attendees, were also included in the present study. Eligible candidates were community-dwellers (i.e. not in residential care such as nursing homes/long-term care facilities), aged 64 years and older and in any stage of health. Exclusion criteria included history of alcoholism, participation in investigational medication trials in the previous month and advanced organic disease (precluding longitudinal follow-up). Two subjects were excluded from the analyses due to unrealistic reported values for daily energy intakes (>4000 kcal).

3.2 Ethical Considerations

Written informed consent was obtained from all subjects, in accordance with local Clinical Research Ethics Committee of the Cork Teaching Hospitals. This study complies with the guidelines set out in the Declaration of Helsinki and those of the Cork Research Ethics Committee, Ireland.

3.3 Data Collection

The Mini-Mental State Examination (MMSE) (Folstein *et al.,* 1975) was used to assess cognitive function. This test covers various aspects of cognitive function, including orientation to time and place, naming, repeating, writing, copying, instantaneous recall, short-term memory, backward spelling. Demographic characteristics of participants including age, gender, body mass index (BMI), diabetes mellitus, hypertension, depression and smoking status were used as covariates in this data analysis. BMI was calculated as weight (kg)/height (m^2). The Mini Nutritional Assessment (MNA) was used to identify subjects at risk of malnutrition (Vellas *et al.,* 1999).

3.4 Dietary data

Dietary data were collected by means of a semi-quantitative food frequency questionnaire (FFQ) administered by trained research nursing personnel. The FFQ was an amended version of that used by the European Prospective Investigation into Cancer (EPIC) study (Riboli & Kaaks, 1997) and validated for use in the Irish population (Harrington *et al.,* 2008). The questionnaire assessed habitual dietary intakes of 147 single food items/beverages. The frequency of intake was measured using ten categories ranging from 'never' to 'six times a day or more'. In order to estimate the number of servings from each food group consumed on a daily basis, each frequency option in the FFQ was converted to a single daily serving. Recently derived, population-based, gender-specific portion sizes for community-dwelling elderly subjects (65-75 yrs and ≥75 yrs) were applied to each FFQ item (Wrieden & Barton, 2006). A nutrient composition database was established for the ELDERMET FFQ using information compiled from the UK Food Standards Agency (FSA) nutrient databank (Food Standards Agency, 2002), with additional information from the Irish Food Consumption database (Black *et al.,* 2011) and manufacturers' information. The FSA food compositional databank was the primary nutrient data source used. This information was used to calculate mean daily nutrient (carbohydrate) intakes. In order to assess diet quality, a healthy food diversity (HFD) index was calculated for each subject as outlined previously (Claesson *et al.,* 2012; Drescher *et al.,* 2007). Higher values of a HFD reflect a healthier, more diverse diet.

GI values (glucose referenced) were assigned to all FFQ items from the International Table of Glycemic Index (Atkinson *et al.,* 2008). An average GI value was assigned to FFQ items consisting of multiple food types (e.g. the 'white bread' category, which includes rolls, panini and ciabattas) based on the median of a range of appropriate foods. In cases where a GI value was not available for a particular food item, values for the most similar food type were assigned. Foods with a low carbohydrate content (e.g. meats, fish, fats), with no corresponding GI value were assigned a default value of 50, as previously described (Flood *et al.,* 2006). The sum total GL of each subject's daily diet was calculated by multiplying the daily carbohydrate intake (g) (from each FFQ item) by the GI value. The GI of each individual's diet was then calculated by dividing GL by the total carbohydrate intake, as described previously (Burger *et al.,* 2011). GI

and GL values were adjusted for energy intakes by dividing absolute GI/GL by total energy intake (kcal) and amounts expressed per 2000 kcal, as described previously (Livesey *et al.,* 2013)

3.5 Identification of dietary patterns

Hierarchical clustering with Euclidean distance and Ward's linkage was conducted using the logged number of daily servings reported from each food/beverage item on the FFQ. The tree was cut at an appropriate height to provide 5 clusters.

3.6 Statistical Methods

Statistical analyses were performed using R (version 2.13.2) and SPSS^{TM} (version 18, Chicago, IL, USA) software packages. Differences between means and medians were evaluated using the Independent-samples t-test/one-way analysis of variance (ANOVA) and the Mann Whitney/Kruskal Wallis test, as appropriate. Differences between categorical distributions were evaluated using chi-square test or Fisher's exact test. Logistic and Poisson regression were used to examine the relation between GI and GL with MMSE score. These models included adjustments for age (continuously, in 1 year increments), sex (male, female), diabetes mellitus (no, yes; medically diagnosed), healthy food diversity (HFD; continuously, in 0.01 point increments), hypertension (no, yes; medically diagnosed) smoking (never, former, current), BMI (continuously, in 1kg/m^2 increments) and the Mini Nutritional Assessment (MNA; continuously in 1 point increments). For Poisson regression, the number of MMSE errors (30 – x; where x is the MMSE score of the patient and 30 is the maximum MMSE score) was used to generate a Poisson distribution of increasing cognitive decline. For logistic regression analyses, two different cut-off points were used to define cognitive impairment, the clinical threshold of less than 24 and the optimal threshold of less than 28, as defined by O'Bryant *et al.* (2008). They showed that the clinical MMSE cut-score of 24 yielded a moderate estimate of sensitivity with a high specificity for defining cognitive impairment in the clinical setting (O'Bryant *et al.,* 2008). However, the optimal cut-off score of 28 was found to yield a much better balance of sensitivity and specificity (O'Bryant *et al.,* 2008). For both regression models, a regression coefficient (RC) was calculated per one unit increase in standard deviation (SD), where appropriate. For logistic regression, the odds ratio associated with each explanatory variable was

calculated as the exponential function of the regression coefficient (e^{RC}). For Poisson regression, the incidence rate ratio associated with each explanatory variable was calculated as the exponential function of the regression coefficient (e^{RC}). A *P* value of <0.05 was considered as statistically significant.

4.0 Results

Selected demographic and anthropometric characteristics of study participants are shown in Table 1. The mean age of the sample was 75 years. Women had a lower body weight and shorter stature than men. The mean BMI was 28 kg/m^2 with the majority of subjects having a BMI greater than 25. Based on the MNA, the majority of subjects were classified as having an adequate nutritional status (MNA≥24). A very low proportion (6%) of subjects were current smokers, while just under half (47%) of subjects were former smokers. The mean MMSE score was 27 with 8.2% and 39.4% of subjects being classified as clinically cognitively impaired (MMSE<24) and mildly cognitively impaired (MMSE<28), respectively. Just under half (40%) of subjects had hypertension, while 11% of subjects were diagnosed as diabetics.

We identified 5 groups in this population based on hierarchical cluster analyses (Fig. 1). A total of 60 subjects (29%) were in cluster 1, 44 (21 %) in cluster 2, 34 (16%) in cluster 3, 51 (25%) in cluster 4 and 19 (9%) in cluster 5. Median food group consumption (servings/day) for each cluster is shown in Table 2. Meat products, chicken, eggs, potatoes/chips, dairy spreads, alcohol, brown bread and miscellaneous food items (jams, sauces etc.) were consumed at similar levels across all five clusters (*P*>0.05). Clusters 1 and 2 were characterised by high intakes of red meat, white bread and low intakes of fruit and vegetables, with cluster 2 having the highest intakes of desserts/sweets, high fat dairy and soft drinks across all five clusters. As cluster 1 had lower intakes of desserts/sweets, high fat dairy and soft drinks relative to cluster 2, these clusters were labelled the 'low-fat Western' and 'Western' dietary patterns, respectively. Cluster 3 was characterised by high intakes of red meat, butter, fresh soups and moderate-high intakes of fruit and vegetables, salad dressings, desserts/sweets and white bread. This cluster was labelled the 'traditional Irish' dietary pattern. Clusters 4 and 5 had the highest intakes of fruit and vegetables, salad dressings, fresh fish, probiotics, pasta/rice, low-fat dairy and the lowest intakes of red meat and white bread across all five clusters. However, as cluster 4 had lower intakes of butter, salad

dressings, desserts/sweets and high fat dairy relative to cluster 5, these clusters were labelled the 'low-fat prudent' and 'prudent' dietary pattern, respectively.

There was a significant difference in the HFD score, GI and GL across all dietary patterns (Fig. 2), with the 'Western' dietary patterns having a lower HFD score and a higher GI and GL than the 'prudent' dietary patterns ($P<0.05$). There was no significant difference in MNA score between the clusters (data not shown; $P>0.05$). Elderly subjects consuming the 'prudent' dietary patterns had a higher MMSE score than those consuming the 'Western' dietary patterns ($P<0.05$), indicating better cognitive function (Fig. 2).

Results of the multivariable-adjusted Poisson regression models that tested associations between GI, GL and cognitive function (number of MMSE errors) are shown in Table 3. After controlling for potential confounders (age, gender, diabetes, HFD, hypertension and MNA), statistically significant associations were observed between GI/GL of the diet and the number of MMSE errors ($P<0.05$), indicative of cognitive decline. Indeed, for every one unit increase in glycaemic index, the incident rate for the number of MMSE errors increased 1.03 times (by 3%), while for every one unit increase in glycaemic load, the incident rate for the number of MMSE errors increased 1.01 times (by 1%). Furthermore, age, hypertension and diabetes were also associated with the number of MMSE errors ($P<0.05$), indicative of cognitive decline, while MNA score was inversely associated with the number of MMSE errors. Further adjustments for body mass index (BMI) and smoking did not effect these results (data not shown). Results of the multivariable-adjusted logistic regression models that tested associations between GI, GL and mild cognitive impairment, and clinically significant cognitive impairment (MMSE <28 and MMSE < 24, respectively) are shown in Table 4 and Table 5. Using the MMSE score cut-off point of 28, there was a significant association between GI ($P<0.05$) and GL ($P<0.01$) with mild cognitive impairment (Table 4). Increasing the glycaemic index by one unit increased the odds of being cognitively impaired by a factor of 1.10 (by 10%), while increasing the glycaemic load by one unit increases the odds of being cognitively impaired by a factor of 1.03 (by 3%). Age and diabetes were positively associated with cognitive impairment, while MNA score was inversely associated with cognitive impairment in this elderly group ($P<0.05$). Using the MMSE cut-off point of 24, there was a significant association between GL ($P<0.05$) and

clinically significant cognitive impairment but other covariates failed to show significance, despite known associations (Table 5).

5.0 Discussion

In this cross-sectional study of elderly Irish men and women we defined five dietary patterns which were associated with divergent food intakes: a 'Western' diet, a 'low-fat Western' diet, a 'traditional Irish' diet, a 'prudent' diet and a 'low-fat prudent' diet. The dietary patterns identified in this study show similarity to those identified in other elderly cohorts (Hamer *et al.*, 2010; Ledikwe *et al.*, 2004; Pryer *et al.*, 2001) and a cohort of middle-aged Irish adults (Villegas *et al.*, 2004). Similar to the 'traditional Irish' and the 'Western' dietary patterns identified in the present study, Villegas *et al.* (2004) identified a 'traditional' diet having high intakes of beverages (non-alcoholic), white bread and refined cereals, butter, whole milk and dairy products, desserts and sweets, and the lowest intakes of fish and alcohol. They also identified a 'prudent' diet, characterised by high intakes of pasta, rice, brown breads, unrefined cereals, poultry, fish, low-fat dairy, salad dressings, fruit and vegetables, and by low intakes of chips, white bread, refined cereals, butter, high-fat dairy, meat, meat products and sweets. Unlike the findings of Villegas *et al.* the present study did not identify an 'alcohol and convenience foods' dietary pattern.

The 'Western' dietary patterns in the present study had a lower HFD score and had a higher GI and GL compared to the 'prudent' dietary patterns. Moreover, subjects consuming the 'prudent' dietary pattern had higher MMSE scores, indicating better cognitive function compared to subjects consuming a 'Western' diet. In agreement with the present study, a number of studies have reported that healthy dietary patterns (with higher intakes of fruit, vegetables, fish, nuts and legumes and lower intakes of meats, high-fat dairy and sweets) are associated with better cognitive function (Akbaraly *et al.*, 2009b; Gu & Scarmeas, 2011; Scarmeas *et al.*, 2006) while Western dietary patterns (with higher intakes of meat, high fat dairy, desserts/sweets, refined cereals and lower intakes of fruit and vegetables) are associated with poorer cognitive function (Francis & Stevenson, 2013; Kanoski & Davidson, 2011; Torres *et al.*, 2012). Scarmeas *et al.* (2006) reported that higher adherence to the Mediterranean diet (high in fruit, vegetables, cereals, legumes, fish, olive oil and low in meat, poultry and saturated fats) was associated with a decreased risk of cognitive decline and Alzheimer's disease in a

non-demented, multiethnic cohort of elderly subjects. These results were supported by data of Akbaraly *et al.* (2009b) who showed that a diet rich in fruit, vegetables and fish was associated with lower odds of cognitive deficit, while greater odds of cognitive deficit were associated with a diet rich in processed meat, chocolate, desserts, fried food, refined grains and high fat dairy products in a middle-aged cohort. Similarly, Torres *et al.* (2012) found that a higher intake of processed foods (desserts, biscuits, potatoes, refined grains, fried foods, high fat dairy, snacks, high fat takeaway, chocolate and sweets, processed meat and fish, sugar beverages and red meat) was associated with reduced memory and impaired executive function in a group of older people with mild cognitive impairment.

Initial analysis of the data showed a strong significant association between cognitive function and the HFD of the diet (data not shown). However, after further adjustment, and when nutritional status was taken into account, the HFD of the diet failed to reach significance in all but one statistical model. This result demonstrates the importance of adequate nutrition for the maintenance of cognitive health and suggests that the association between healthy dietary patterns and cognition may not be as significant as previously reported, due to the strong association between nutritional status and cognitive function.

GI and GL were inversely associated with cognitive function in this elderly Irish population group; this association remained significant after adjustment for a large number of co-factors using both logistic and Poisson regression. To the best of our knowledge, this is the first study to directly examine the relationship between GI/GL and cognitive function in the elderly. The results of this study are supported by several other studies which have found associations between insulin resistance (Neumann *et al.*, 2008; Zhong *et al.*, 2012), blood glucose levels (Crane *et al.*, 2013; Ravona-Springer *et al.*, 2012) impaired glucose tolerance (Lamport *et al.*, 2009) and diabetes (Gregg *et al.*, 2000; Strachan *et al.*, 2011), and cognitive function in the elderly. A previous prospective study found that glycaemic load was not associated with increased risk of Alzheimer's disease (Luchsinger *et al.*, 2007).

The results of the present study may be explained by a number of hypotheses, including hyperglycemia and hyperinsulinemia. It is well established that high GI diets lead to increased blood glucose levels (Ludwig, 2002). Crane and colleagues found that higher

glucose levels were associated with an increased risk of dementia in populations with and without diabetes (Crane *et al.,* 2013). The brain uses glucose, almost exclusively, as its source of energy, thus making glucose regulation central to brain function (Kimura, 2003). A rodent study previously showed a lack of protective adaptation of the blood brain barrier to limit glucose transfer into the brain (Jacob *et al.,* 2002). Therefore the brain tissue can potentially be exposed to elevated glucose levels, possibly leading to tissue damage, including brain lesions (Trone *et al.,* 2009) which have been shown to play a role in the development of cognitive impairments in elderly subjects (Frisoni *et al.,* 2007). High GI diets are also known to lead to hyperinsulinemia (Ludwig, 2002) which is associated with decreased cognitive function and dementia in healthy elderly subjects (Kalmijn *et al.,* 1995; Zhong *et al.,* 2012). Insulin secreted by the pancreas can cross the blood-brain barrier where it can exert region-specific effects on glucose metabolism (Neumann *et al.,* 2008). Results from human and animal studies suggest that insulin may also have a direct effect on the brain and cognition (Gispen & Biessels, 2000; Ott *et al.,* 2012). Neurotransmitter release, neuronal-outgrowth, neuronal survival and synaptic plasticity are all directly modulated by insulin and therefore, hyperinsulinemia may have a detrimental effect on the function and survival of neurons (Neumann *et al.,* 2008).

In line with previous findings (Gregg *et al.,* 2000; Saka *et al.,* 2010; Strachan *et al.,* 2011; Waldstein, 2003), this study supports an association between cognitive function and other factors, namely age, hypertension, diabetes and nutritional status. The present study has several methodological strengths, one of which is the availability of extensive adjustment data. Careful control for potential confounders is important in an observational setting to limit biases that may arise, since biological and other medical characteristics may influence both dietary intake and cognitive function. Indeed the association between GI/GL and cognitive function remained significant after controlling for age, gender, diabetes, hypertension, HFD, smoking MNA and BMI. Although a number of subjects in the present study were classified as cognitively impaired using the standard MMSE cut score of 24, a much larger proportion had mild cognitive impairment as defined by a MMSE score of 28. While the MMSE cut-score of 24 is useful in a clinical setting due to its high specificity, it has a very low sensitivity score. A previous study has found that a MMSE cut-off score of 28 has an improved balance of specificity and sensitivity (O'Bryant *et al.,* 2008).

In conclusion, the present results suggest that the GI/GL of the diet is inversely associated with cognitive performance in the elderly and may partly explain the relationship between the 'Western' dietary pattern and cognitive impairment which has been highlighted in this and previous studies (Francis & Stevenson, 2013; Kanoski & Davidson, 2011; Torres *et al.*, 2012). The results of this cross-sectional study generate a new hypothesis and contribute to a better understanding of the link between nutrition and cognition. Additional prospective investigations, intervention and mechanistic studies are needed in order to establish a relationship between a low GI/GL diet and cognitive decline in the elderly, to elucidate the underlying biological mechanisms. Given the prevalence and clinical significance of dementia, associations between the GI/GL of the diet and cognitive function could have considerable clinical and public health relevance and warrant further investigation.

Table 1. Descriptive characteristics of the study's participants (*n* 208) by gender

Characteristics	**Males** (*n* 94)		**Females** (*n* 114)	
	Mean	**SD**	**Mean**	**SD**
Age (yrs)	75.4	6.5	74.8	6.7
Weight (kg)	83.2	14.3	69.3***	15.1
Height (cm)	170.4	7.2	158.5***	5.1
BMI (kg/m^2)	28.5	4.1	27.6	6.1
BMI categories *(%)*				
Underweight (<18.5)	1		2	
Normal (18.5-24.9)	18		32	
Overweight (25-29.9)	45		34	
Obese (≥30)	36		32	
MNA score (0, 30)	26.0	2.0	25.8	2.2
Nutritional status *(%)*				
Normal Nutritional Status (MNA ≥24)	90		87	
At risk of malnutrition (MNA 17-23.5)	10		13	
Malnourished (MNA<17)	0		0	
Smoking status *(%)*				
Never	41		52	
Former smoker	54		42	
Current	5		6	
MMSE score (0, 30)	27.4	2.7	27.5	2.8
Cognitive Impairment (%)				
MMSE <24	7.5		8.8	
MMSE <28	39.4		39.5	
HFD score	0.41	0.10	0.42	0.09
Diabetics *(%)*	15		8	
Insulin-dependent	2.1		0.9	
Non-insulin dependent	12.8		7.0	
Hypertension *(%)*	43		39	
Glycaemic Index (per 2000kcal)	58.99	4.06	57.04***	3.72
Glycaemic Load (per 2000kcal)	146.78	20.15	142.42	18.34

Values represent mean and standard deviation (SD) or percentages (%)

BMI, body mass index; HFD, healthy food diversity; MMSE, mini-mental state examination; MNA, mini-nutritional assessment

***P ≤ 0.001: comparisons between males and females (Independent-samples t test).

Table 2. Food group intakes (servings/d) by dietary pattern clusters

Food group	Cluster 1 'Low-fat Western'		Cluster 2 'Western'		Cluster 3 'Traditional Irish'		Cluster 4 'Low-fat prudent'		Cluster 5 'Prudent		*P* value
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	
High consumption foods											
Red meat	0.61	0.44- 0.86	0.70	0.45-0.87	0.70	0.51-1.03	0.43	0.24-0.64	0.40	0.23-0.70	0.000
Fish	0.15	0.02-0.28	0.15	0.04-0.28	0.19	0.14-0.31	0.29	0.18-0.46	0.44	0.15-0.57	0.000
Fruit	2.23	1.18- 3.46	1.41	0.39-2.81	2.79	1.92-4.20	3.30	1.71-4.58	3.42	2.06-4.39	0.000
Vegetables	2.13	1.48-3.64	1.98	1.61-2.58	3.85	2.89-6.14	3.60	2.48-5.28	4.35	2.76-6.67	0.000
Butter	0.00	0.00-1.01	0.29	0.00-2.00	1.00	0.01-2.00	0.00	0.00-1.00	0.29	0.00-2.00	0.009
High fat dairy	0.30	0.02-1.01	1.23	0.58-2.89	0.73	0.15-2.08	0.72	0.14-1.29	1.06	0.61-2.86	0.000
Low fat dairy	0.01	0.00-1.01	0.00	0.00-0.71	0.03	0.00-1.00	0.29	0.00-1.14	0.32	0.00-2.58	0.046
Desserts/sweets	1.64	0.63-3.15	4.82	3.10-9.11	3.43	1.74-5.81	1.14	0.38-2.21	3.00	1.25-4.31	0.000
White bread	0.79	0.04-2.02	1.03	0.30-2.08	0.48	0.17-2.04	0.10	0.03-0.30	0.49	0.29-1.14	0.000
Wholegrain breakfast cereals	1.00	0.00-1.00	1.00	0.04-1.00	1.00	0.11-1.00	1.00	1.00-1.00	1.00	0.71-1.30	0.046
Low consumption foods											
Chicken products	0.00	0.00- 0.01	0.00	0.00-0.03	0.00	0.00-0.01	0.00	0.00-0.00	0.00	0.00-0.03	0.009
Fish products	0.03	0.00-0.14	0.01	0.00-0.03	0.03	0.01-0.14	0.00	0.00-0.01	0.01	0.00-0.14	0.000
Soft drinks	0.01	0.00-0.29	0.14	0.00-0.93	0.01	0.00-0.29	0.00	0.00-0.02	0.02	0.00-0.15	0.005
Savoury snacks	0.00	0.00-0.02	0.00	0.00-0.00	0.01	0.00-0.03	0.00	0.00-0.02	0.17	0.03-0.58	0.017
Refined grain breakfast cereals	0.00	0.00-0.29	0.00	0.00-0.00	0.00	0.00-0.00	0.00	0.00-0.00	0.00	0.00-0.01	0.031
Pasta/rice	0.01	0.00-0.05	0.00	0.00-0.03	0.03	0.01-0.14	0.06	0.02-0.17	0.34	0.20-0.57	0.000
Salad dressing	0.01	0.00-0.15	0.04	0.00-0.29	0.10	0.01-0.30	0.15	0.03-0.29	0.31	0.21-0.61	0.000
Processed soups	0.03	0.00-0.29	0.02	0.00-0.25	0.01	0.00-0.14	0.00	0.00-0.03	0.01	0.00-0.28	0.023
Ready meals	0.01	0.00-0.04	0.01	0.00-0.03	0.02	0.01-0.09	0.01	0.00-0.06	0.07	0.03-0.16	0.005
Fresh Soups	0.00	0.00-0.06	0.10	0.00-0.29	0.29	0.12-0.73	0.14	0.00-0.29	0.14	0.00-0.29	0.000
Probiotics	0.00	0.00-1.00	0.00	0.00-0.03	0.03	0.00-0.71	0.14	0.00-1.00	0.71	0.14-1.00	0.003

Values represent median and interquartile ranges (IQR). Differences between dietary patterns were evaluated using the Kruskal Wallis test. Other food groups analysed incl. meat products, chicken, eggs, potatoes, chips, dairy spreads, alcohol, brown bread and miscellaneous food items (jams, sauces etc) showed no significant difference (P>0.05) in intakes between the dietary pattern clusters and were excluded from the table

Table 3. Poisson regression tests of associations between A) dietary glycaemic index and B) dietary glycaemic load (adjusted to 2000kcal) with MMSE score (adjusted for age, gender, diabetes HFD, hypertension and MNA score)

	Parameters	Regression Coefficient	RC x SD	e^{RC}	Std. Error	Z-value	*P*-value
A)	Intercept	-0.37		0.69	1.11	-0.34	*ns*
	Glycaemic index	**0.03**	**0.11**	1.03	**0.01**	**2.39**	***0.017***
	Age	0.03	0.19	1.03	0.01	4.33	*0.000*
	Gender (female)	0.13		1.14	0.09	1.40	*ns*
	Diabetes	0.48		1.61	0.12	4.06	*0.000*
	Hypertension	0.24		1.27	0.10	2.44	*0.015*
	HFD	-0.81	-0.08	0.44	0.51	-1.59	*ns*
	MNA	-0.09	-0.19	0.91	0.02	-4.78	*0.000*
B)	Intercept	0.20		1.22	0.84	0.24	*ns*
	Glycaemic load	**0.01**	**0.17**	1.01	**0.00**	**3.87**	***0.000***
	Age	0.03	0.18	1.03	0.01	4.10	*0.000*
	Gender (female)	0.11		1.12	0.09	1.28	*ns*
	Diabetes	0.47		1.61	0.12	4.06	*0.000*
	Hypertension	0.22		1.24	0.10	2.17	*0.030*
	HFD	-0.99	-0.09	0.37	0.49	-2.03	*0.042*
	MNA	-0.09	-0.19	0.91	0.02	-4.81	*0.000*

Regression coefficients (RC) indicate the relative magnitude and direction of the association; A positive RC indicates that the explanatory variable is associated with an increase in MMSE errors, while a negative RC indicates that the explanatory variable is associated with fewer MMSE errors. HFD, healthy food diversity; MNA, Mini Nutritional Assessment; ns, non-significant (P>0.05); RC x SD, regression coefficient per increase with one standard deviation; e^{RC}, exponentiated regression coefficient.

Table 4. Logistic regression tests of associations between **A)** dietary glycaemic index and **B)** dietary glycaemic load (adjusted to 2000kcal) with cognitive impairment (MMSE score<28), adjusted for age, gender, diabetes, HFD hypertension and MNA score

	Parameters	Regression Coefficient	RC x SD	e^{RC}	Std. Error	Z-value	P-value
A)	Intercept	-5.92		0.00	4.07	-1.45	*ns*
	Glycaemic index	**0.09**	**0.37**	1.10	**0.04**	**2.15**	***0.032***
	Age	0.06	0.39	1.06	0.02	2.43	*0.015*
	Gender (female)	0.28		1.33	0.32	0.89	*ns*
	Diabetes	1.13		3.11	0.51	2.23	*0.026*
	Hypertension	-0.15		0.86	0.35	-0.44	*ns*
	HFD	0.57	0.05	1.77	1.80	0.32	*ns*
	MNA	-0.18	-0.37	0.84	0.08	-2.36	*0.019*
B)	Intercept	-3.56		0.03	3.15	-1.13	*ns*
	Glycaemic load	**0.03**	**0.50**	1.03	**0.01**	**2.93**	***0.003***
	Age	0.05	0.36	1.06	0.02	2.23	*0.026*
	Gender (female)	0.23		1.26	0.32	0.72	*ns*
	Diabetes	1.19		3.30	0.51	2.34	*0.019*
	Hypertension	-0.05		0.96	0.36	-0.13	*ns*
	HFD	-0.20	-0.02	0.82	1.73	-0.12	*ns*
	MNA	-0.19	-0.39	0.83	0.08	-2.42	*0.015*

Regression coefficients indicate the relative magnitude and direction of the association; HFD, healthy food diversity; MNA, Mini Nutritional Assessment; ns, non-significant (P>0.05); e^{RC}, exponentiated regression coefficient; RC x SD, regression coefficient per increase with one standard deviation

Table 5. Logistic regression tests of associations between **A)** dietary glycaemic index and **B)** dietary glycaemic load (adjusted to 2000kcal) with cognitive impairment (MMSE score<24), adjusted for age, gender, diabetes, HFD hypertension and MNA score)

	Parameters	Regression Coefficient	RC x SD	e^{RC}	Std. Error	Z-value	*P*-value
A)	Intercept	-6.54		0.00	6.81	-0.96	*ns*
	Glycaemic index	**0.09**	**0.36**	1.10	**0.07**	**1.26**	***ns***
	Age	0.06	0.42	1.07	0.04	1.49	*ns*
	Gender (female)	0.50		1.65	0.58	0.87	*ns*
	Diabetes	1.07		2.91	0.66	1.61	*ns*
	Hypertension	-0.85		0.43	0.60	-1.42	*ns*
	HFD	-3.35	-0.31	0.04	3.28	-1.02	*ns*
	MNA	-0.19	-0.39	0.83	0.12	-1.60	*ns*
B)	Intercept	-6.44		0.00	5.19	-1.24	*ns*
	Glycaemic load	**0.04**	**0.75**	1.04	**0.02**	**2.53**	***0.01***
	Age	0.06	0.42	1.07	0.04	1.49	*ns*
	Gender (female)	0.52		1.68	0.58	0.90	*ns*
	Diabetes	1.14		3.13	0.68	1.69	*0.09*
	Hypertension	-0.86		0.42	0.62	-1.39	*ns*
	HFD	-3.60	-0.34	0.03	3.12	-1.16	*ns*
	MNA	-0.20	-0.43	0.81	0.12	-1.70	*0.09*

Regression coefficients indicate the relative magnitude and direction of the association; HFD, healthy food diversity; MNA, Mini Nutritional Assessment; ns, non-significant (P>0.05); e^{RC}, exponentiated regression coefficient; RC x SD, regression coefficient per increase with one standard deviation

Figure 1. Five dietary clusters revealed through hierarchical clustering with Euclidean distance metric and Ward's linkage. Cluster 1 'low-fat Western =red; Cluster 2 'Western'= blue; Cluster 3 'traditional Irish'= green; Cluster 4 'low-fat prudent' =brown; Cluster 5 'prudent'= magenta. Key nodes in hierarchical tree are circled.

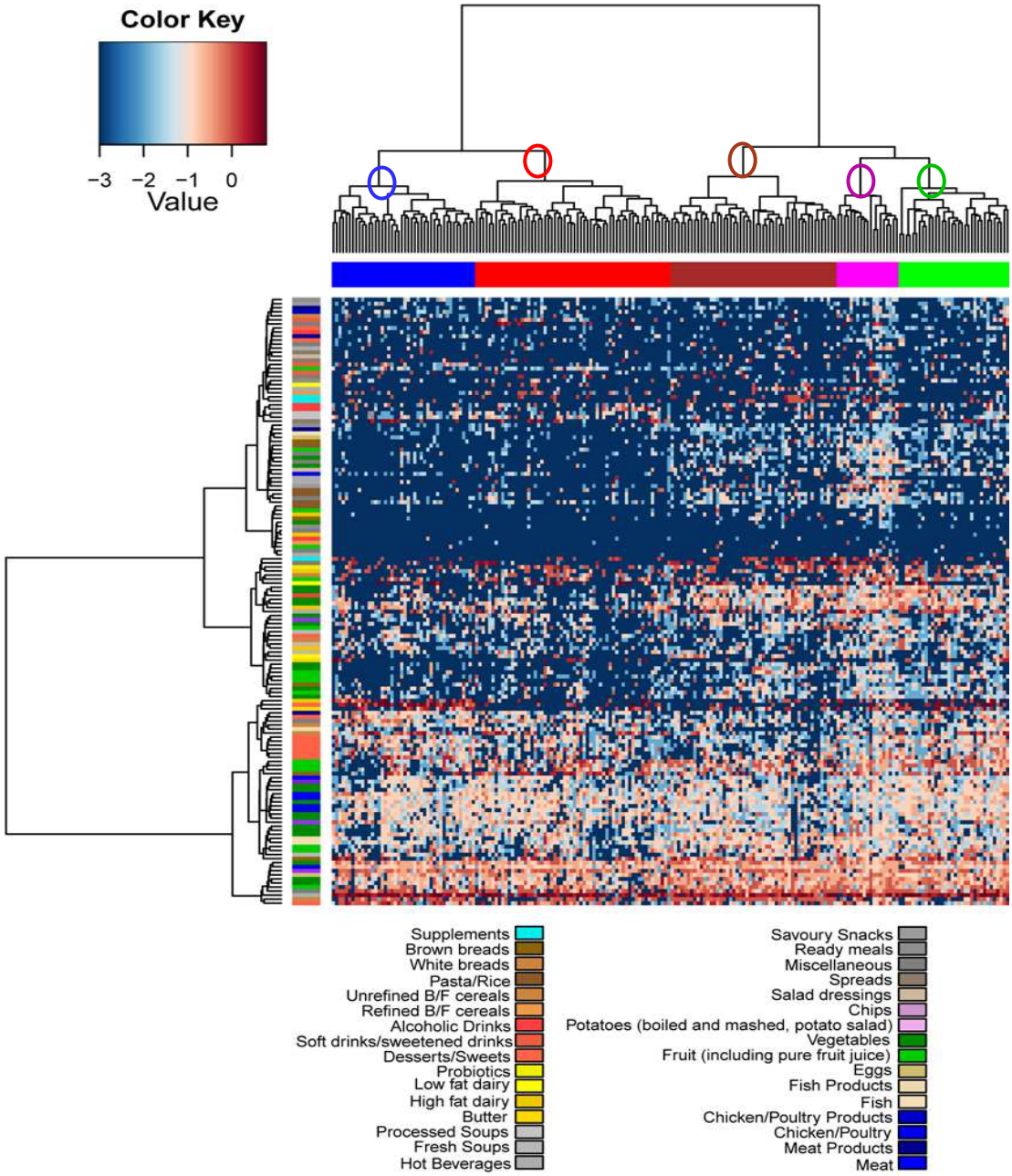
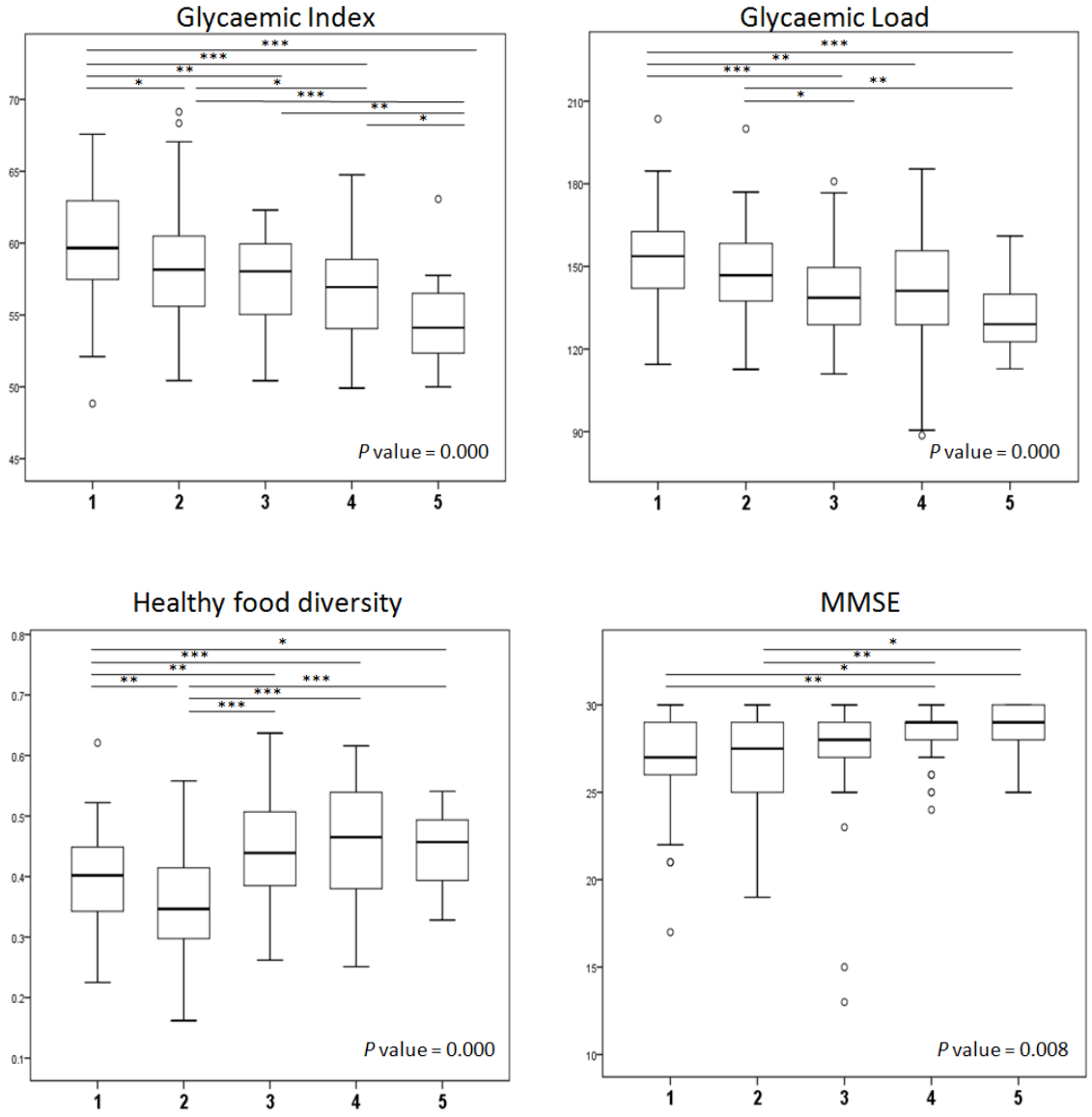


Figure 2. Comparison of **glycaemic index**, **glycaemic load**, **healthy food diversity and MMSE** score, between dietary pattern clusters (1=low-fat Western; 2=Western; 3=traditional Irish; 4=low-fat prudent; 5=prudent)**.** *P* value refers to the Kruskal-Wallis test performed across all five dietary pattern clusters. The Mann-Whitney test was performed for each pair-wise comparison. Open circles indicate outliers. *P≤0.05; ** P≤0.01; ***P≤0.001



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CHAPTER 5

Immunomodulatory effects of 'viable' and 'non-viable' *Bifidobacterium*-fermented milks: IL-10 and TNF-α secretion by human peripheral blood mononuclear cells

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1.0 Abstract

It is well recognised that certain therapeutic microbes, including *Bifidobacterium longum* subsp. *infantis* 35624, exert health benefits on the host. A number of different mechanisms by which they are proposed to improve health have been reported, including modulation of the host's immune response. An area of ongoing debate is whether non-viable forms of probiotic strains have a role to play in conferring benefits to the host by influencing the host's immune system. This study investigated the effect of both 'viable' and 'non-viable' *Bifidobacterium* strains in fermented milk products on the release of the cytokines IL-10 and TNF-α from peripheral blood mononuclear cells (PBMCs) *in vitro*. *B. infantis* 35624, *B. breve* UCC2003, *B. longum* 0103 and *B. longum* 0106, were grown in a fortified whole-milk preparation and subjected to a number of lethal treatments (heat treatment, high pressure treatment and storage at low pH) in order to render the bacterial cells non-culturable. Freshly isolated PBMCs were then cultured in the presence of the lyophilised *Bifidobacterium*-fermented milk (BFM) (containing either 'viable' or 'non-viable' bifidobacteria) at different concentrations. The results show that BFM induces a strain-specific immune response in the PBMC assay. Furthermore, it was found the BFM could induce IL-10 and TNF-α production even after bifidobacterial cells were rendered non-culturable. However, the method used for killing the cells was found to play a significant role in the immune response, with extended storage at 4ºC being the best method for killing cells while maintaining immunomodulatory activity. This indicates that although the product is microbiologically inactive (contains no culturable cells), it may still be able to induce immunological effects and may therefore have beneficial applications in human health.

2.0 Introduction

Probiotics are defined as live microorganisms which when administered in adequate amounts confer health benefits to the host (FAO/WHO, 2002). The most extensively studied and commonly used probiotic organisms are lactobacilli and bifidobacteria (Lammers *et al.,* 2003; Senok *et al.,* 2005) and dairy products are one of the main food vectors presently used for their delivery (Rivera-Espinoza & Gallardo-Navarro, 2010). Nowadays, the medical, health and commercial importance of probiotic products is well recognised and consequently, many products of this kind are available for consumption by both humans and animals. Indeed, the global market for probiotic products was estimated at $24.2 billion in 2011 and is expected to grow to $36.0 billion in 2017 (MarketsandMarkets, 2013).

Probiotics have been shown to play a role in the prevention and treatment of various diarrhoeal conditions as well as inflammatory diseases of the gastrointestinal tract (GIT) (Quigley, 2010; Sanders *et al.,* 2013). Certain therapeutic microbes have been shown to be effective in the relief of symptoms caused by inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) (Quigley, 2007; Sanders *et al.,* 2013). *Bifidobacterium longum* subsp. *infantis* 35624 (*B. infantis* 35624) is a commercial probiotic strain with well known anti-inflammatory properties (McCarthy *et al.,* 2003; O'Mahony *et al.,* 2008) and has been reported, in two separate studies, to relieve many of the most troublesome symptoms of IBS (O'Mahony *et al.,* 2005; Whorwell *et al.,* 2006). Furthermore, *B. infantis* 35624 was also shown, in one of these studies, to normalise the baseline pro-inflammatory cytokine profile in peripheral blood (O'Mahony *et al.,* 2005). A recent study found that oral administration of *B. infantis* 35624 resulted in significantly elevated IL-10 produced by peripheral blood mononuclear cells (PBMCs) as well as increased T regulatory cells, indicating that this strain induces a regulatory immune response in humans (Konieczna *et al.,* 2012). In addition, oral administration of *B. infantis* 35624 has been shown to reduce systemic inflammatory biomarkers in both gastrointestinal and extra-intestinal inflammatory disorders (Groeger *et al.,* 2013).

It has been suggested that the minimum therapeutic dose for probiotics is 10^8-10^9 viable cells per day. This would require probiotic products to contain a minimum 10^6 colony forming units (CFU)/mL in a typical 100 mL delivery product in order to be effective (Kailasapathy and Chin, 2000). However, a problem with the practical application of

probiotics is that live cells in probiotic products will inevitably lose viability and the actual products will contain varying numbers of these cells (Shah *et al.,* 1995). Indeed substantial research and development has been dedicated to processes and agents that can prolong their stability and viability in beverage/food products and remove the need for refrigeration (Ranadheera *et al.,* 2010). There may also be substantial loss of viability of the organisms on passage through the relatively hostile environment of the GIT (Adams, 2010). Concerns have also been raised regarding the potentially detrimental effects of administering these as live agents, particularly to immunocompromised subjects (Kataria *et al.,* 2009).

An area of ongoing debate is whether the non-viable forms of beneficial bacterial strains in formulations have a role to play in conferring benefits to the consumer by influencing the host's immune system (reviewed in: Adams, 2010; Kataria *et al.,* 2009; Taverniti & Guglielmetti, 2011). Studies have proposed that the immunomodulatory effects exerted by non-viable probiotics may be due to their immunostimulatory DNA, cell wall components, peptidoglycan, intra and/or extra-cellular polysaccharide products (Hosono *et al.,* 1997; Lammers *et al.,* 2003; Rachmilewitz *et al.,* 2004) and hence may be dependent on method of preparation (Hiramatsu *et al.,* 2007). The technology described in the literature to render probiotic bacteria non-viable is usually heat treatment (Cross *et al.,* 2004; Hiramatsu *et al.,* 2007; Li *et al.,* 2009; Matsuguchi *et al.,* 2003; Xiao *et al.,* 2002; Zhang *et al.,* 2005). However, because of the possible impact heat treatment has on cellular integrity and protein structure (Lopez *et al.,* 2008) other studies have also used γ-irradiation (Rachmilewitz *et al.,* 2004), UV light (Lopez *et al.,* 2008) or sonication (Hiramatsu *et al.,* 2007).

The present study was based on the hypothesis that *Bifidobacterium*-fermented milks induce a strain-specific immune response and 'non-viable' forms of these *Bifidobacterium*-fermented milks can maintain this immunomodulatory activity. In the present study, fermented milk products containing *B. infantis* 35624, *Bifidobacterium breve* UCC2003 and two *Bifidobacterium longum* strains (0103 and 0106), were investigated for their ability to induce cytokine production (IL-10 and TNF-α) in an *in vitro* model of host response. In addition, the effects of three different methods (heat treatment, high pressure treatment and storage at low-pH) for killing the *Bifidobacterium* strains within the milk preparation were examined. The cytokine

response patterns of the non-viable (i.e. ‘dead’) cell preparations was compared to their viable (i.e. ‘live’) counterparts.

3.0 Materials and Methods

3.1 Preparation of cultures for inoculation into malted milk

The strains used in this study included *B. longum infantis* 35624, *B. breve* UCC2003, *B. longum* NCIMB 41713 (0103) and *B. longum* NCIMB 42020 (0106). All bacterial cultures were maintained in 40% glycerol and stored at -80°C. The -80°C strains were inoculated (2% v/v) into modified de Man, Rogosa and Sharpe medium (mMRS) comprising MRS medium (Oxoid, Hampshire, UK) supplemented with 0.05% (w/v) cysteine-HCl and incubated for 24 h at 37°C under anaerobic conditions (anaerobic jars with Anaerocult A gas packs; Merck, Darmstadt, Germany). At the end of incubation, the cultures were sub-cultured (2% v/v) into 150 mL mMRS and reincubated for a further 24 h.

3.2 Milk preparation

The fermentation substrate consisted of whole milk fortified with 2% (w/w) skimmed milk powder, 2% (w/w) sucrose and 2% (w/w) malt extract. Following preparation, the fermentation substrate was autoclaved at 115°C for 10 min in 500 mL aliquots. The substrate was allowed to cool to 37°C before being inoculated (2% v/v) with a fresh overnight culture of each strain and incubated for 15-16 h at 37°C. A milk control (i.e. whole milk fortified with 2% skimmed milk powder, 2% sucrose and 2% malt extract) that was not inoculated with *Bifidobacterium* was also included. Six independent batches of each *Bifidobacterium*-fermented milk (BFM) and milk control were used for subsequent experiments (3 independent batches for storage (4ºC and 37ºC) at low pH (pH 4.0-4.6) and 3 independent batches for heat and high pressure treatment).

3.3 Lethal Treatment

3.3.1 Storage at low pH

Following fermentation the BFM preparations (containing ~10^8 CFU/mL of stationary phase cells) and the milk control were divided into approximately 250 mL volumes in sterile plastic containers (Sarstedt, Wexford, Ireland). These were then stored at 4°C or 37°C over a period of time. Viable cell counts and pH were recorded prior to storage and at appropriate intervals over a number of days/weeks depending on the specific

strain and the storage temperature. The pH changes were monitored using a pH meter, model R720 (Reagecon, Co. Clare, Ireland), with a calibrated electrode (Schott, Mainz, Germany).

3.3.2 High pressure (HP) treatment

Aliquots (30 mL) of the BFM preparations and the milk control were dispensed into plastic vacuum bags and vacuum packed. Pressure treatments were carried out using a Stansted Fluid Power Iso-Lab 900 High Pressure Food Processor (Stansted Fluid Power Ltd., Stansted, Essex, UK). The medium for hydrostatic pressurization was a 9:1 mixture of ethanol and castor oil. Samples were subjected to HP in the range 300 - 600 MPa with holding times of 5 min at ambient temperature. Adiabatic heating on compression resulted in an increase in the temperature of the processing fluid (by up to 15°C at 600 MPa). Viable-BFM samples were held at atmospheric pressure at ambient temperature for the duration of the treatment and plated together with HP treated samples for determination of the number of colony forming units (CFU) in each sample.

3.3.3 Heat treatment

Aliquots (12 mL) of the BFM preparations and milk control were dispensed into sterile test tubes and subjected to high temperature treatment. Samples were thermally treated in a water bath for 1 min at 72°C. In order to accurately determine the length of time the cells were exposed to the test temperature a thermometer was placed in a control test tube which contained 10 mL of BFM and timing commenced once the thermometer reached the desired temperature. Samples were immediately cooled for 15 min on ice. Viable-BFM samples were held at ambient temperature for the duration of the treatment (as a temperature control for the heat treatment), cooled on ice and plated together with heat-treated samples in order to determine cell viability.

3.4 Determination of culture viability and quality control

3.4.1 Determination of culture viability

For the enumeration of culturable microorganisms, aliquots of milk were serially diluted in one-quarter strength Ringer’s solution (Merck). The number of colony forming units (CFU) of *Bifidobacterium* was determined by spread plating the appropriate dilutions in

duplicate on Reinforced Clostridial Agar (RCA, Oxoid) supplemented with 0.05% cysteine-HCl. RCA plates were incubated at 37°C for 48 h under anaerobic conditions (anaerobic jars with Anaerocult A gas packs). Contamination levels of the samples were also monitored. Undiluted samples were routinely spread plated on Yeast Extract Glucose Chloramphenicol agar (Merck) for yeast and mould determination (incubated aerobically at 30°C for 5 d); Baird-Parker agar + 1% (v/v) egg yolk tellurite emulsion for determination of staphylococci (incubated at 37°C for 24-48 h), Violet Red Bile (VRB, Merck) agar for determination of coliforms (incubated at 37°C for 24 h) and Tryptone Soy Agar (TSA, Oxoid) + 1% (w/v) yeast extract (Merck) for determination of contaminating aerobes (incubated at 30°C for 72 h followed by 48 h at 37°C). Bacterial counts are reported as $\log_{10}$CFU mL^{-1}.

3.4.2 Quality control – identification/confirmation of strains by polymerase chain reaction (PCR).

Following enumeration of viable *Bifidobacterium* on RCA plates, a selection of colonies of each strain were picked and sub-cultured in mMRS medium for 24 h. A 1 mL aliquot was transferred to a 2 mL stock bottle and centrifuged to pellet the cells. Total DNA was isolated from the pellets using Extract-N-Amp^{TM} Tissue PCR Kit (Sigma). A strain specific 'fingerprint' (based on EPS genes) has previously been generated for each strain by Alimentary Health, Cork, Ireland. Each strain was screened for the presence/absence of a selection of these genes. Identification of samples was confirmed when the expected profiles were observed. PCR reactions were performed using GoTaq Green Master Mix (Promega (M7122). Primers were obtained from MWG Biotech (Eurofins, Germany). PCR conditions were as follows: 94°C for 4 min; 29 cycles of 94°C for 45 sec, 52-56°C (adjusted for annealing temperature of individual primer pairs) for 45 sec and 72°C for 45 sec.

3.5 Freeze drying

Samples were placed in sterile Petri dishes and spread so as to obtain a film of approximately 0.5 cm thickness. The Petri dishes were stored on metal trays and placed in a -80°C freezer prior to freeze drying. Frozen samples were then freeze dried in a VirTis Genesis freeze drier, model 25ES (VirTis, New York) with a standard programme. Following freeze-drying, the powders were then ground in a sterile

stomacher bag and transferred immediately to sterile bottles and capped under atmospheric conditions. Freeze dried powders were stored at -20°C until use. The freeze-dried samples were resuspended in Ringers solution, diluted and subsequently plated. The number of culturable cells after freeze-drying was determined as $\log_{10}$CFU g^{-1}.

3.6 Determination of membrane integrity and total cell counts of freeze dried powders - LIVE/DEAD staining

3.6.1 Extraction of bacterial cells from milk samples

For the recovery of bacteria, 1 mL of the 1/10 (w/v) diluted freeze dried samples were thoroughly mixed in a microcentrifuge tube with 0.5 mL of a milk clearing solution [0.15M N-(2-acetoamido)imino-diacetic acid (ADA, chelating agent), 0.5% (w/v) Triton X-100 (non-ionic detergent)] (McClelland and Pinder, 1994). The tubes were then centrifuged at 12,000 *g* for 5 min. After centrifugation a cream layer was formed on the top of a clear supernatant fluid, and a thick pellet was formed at the bottom. Both the cream layer and the supernatant were carefully removed leaving the pellet containing the microorganisms and residual milk particles behind.

3.6.2 Staining of cells

Staining was carried out using a LIVE/DEAD® BacLight™ bacterial viability kit (Molecular Probes, Leiden, The Netherlands) according to the manufacturer's instructions. The LIVE/DEAD stain consists of a mixture of SYTO 9 and propidium iodide (PI) nucleic acid stains; these stains differ in their spectral characteristics and in their ability to penetrate the membranes of healthy cells. SYTO 9 stains bacteria with intact membranes a fluorescent green whereas bacteria with damaged membranes are stained red on uptake of PI. Briefly, the pellet containing the extracted microorganisms was resuspended in a final volume of 10 mL using a sterile 0.85% NaCl solution. A 10 µL sample was then added to 977 µL 0.85% NaCl in a flow cytometry analysis tube. This was followed by 1.5 µL of SYTO 9 nucleic acid stain and 1.5 µL of PI stain. Samples were incubated for 15 min at room temperature and protected from light. Following staining, 10 µL of the microsphere standard beads (to facilitate counting) was added. The tubes were mixed well and analysed by flow cytometry (FCM) using a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany) and CellQuest

Pro data analysis software (BD Biosciences). Forward scatter (FSC), side scatter (SSC) and two fluorescent signals [SYTO-9-FL1 channel (green) and PI-FL3-channel (red)] were measured as per manufacturers' instructions.

3.7 Peripheral blood mononuclear cells (PBMC) isolation and cytokine induction assay

BFM samples were assayed using the PBMC cytokine induction assay. For each batch of BFM, PBMCs were isolated from peripheral blood of 3 healthy volunteers. Briefly, blood was taken directly into sterile EDTA-containing vacutainers. Mononuclear cells were isolated from blood by density gradient centrifugation (1.077 $g\,mL^{-1}$, Histopaque®, Sigma, Poole, Dorset, U.K.). After two washing steps, PBMCs were resuspended in complete media consisting of Dulbecco's Minimum Essential Medium (DMEM; Gibco Life Technologies), 1% Penicillin Streptomycin (Sigma) and 10% Foetal Calf Serum (Sigma) at 1 x 10^6 cells/mL and 200 µL aliquots were transferred to 96-well flat-bottomed plates. Aliquots of lyophilised BFM samples (100 mg) were resuspended 1/10 (w/v) in Dulbecco's phosphate buffered saline (PBS; Sigma) and then serially diluted. PBMC's were stimulated for 48 h at 37°C in 5% CO_2 atmosphere with 20µL aliquots of the different concentrations of BFM samples and malted milk control (final concentrations: 5 mg/mL, 2.5 mg/mL and 1 mg/mL which equated to a bacterial cell: PBMC ratio of 50:1, 25:1, 10:1). This range of concentrations was used to determine the dose which induced the maximum cytokine secretion. In each assay, a positive control, CH2007 (commercial product - lyophilised, *B. infantis* 35624 provided by Chr. Hansen), for which the cytokine profile is well established, was used to stimulate the PBMCs. Furthermore, a negative control (phosphate buffered saline) with no stimuli was also added to the PBMC cells. After stimulation, cell culture supernatants were harvested by centrifugation and stored at -80°C until cytokine levels were quantified.

PBMC supernatants were thawed at 4ºC and analysed for cytokine (IL-10 and TNF-α) levels using Meso Scale Discovery (MSD; Gaithersburg, Maryland, USA) assays according to the manufacturer's protocol and analyzed on a SECTOR 2400 instrument (MSD). All experiments were performed in triplicate.

3.8 Statistical analysis

Data are expressed as means and standard errors or medians and interquartile range, as appropriate. Values are an average of triplicate independent experiments unless stated otherwise. All data were analysed using Graphpad Prism version 5.1 (GraphPad Software Inc.). Statistical comparisons were made with a one- or two-way ANOVA with a Bonferonni correction. For non-parametric data, statistical comparisons were made using the Kruskal Wallis test with a Dunn correction. Statistical significance was set at $P<0.05$ and significance levels were defined as *P<0.05, **P<0.01 and ***P<0.001.

4.0 Results

4.1 Viability of bacteria during storage at 4°C and 37°C (pH effect) and following high pressure (HP) and heat treatment (HT)

The BFM preparations had a pH of approximately 4.5-4.6, and contained 10^8-10^9 CFU mL^{-1} following 15-16 h fermentation at 37°C. The results indicate that storage temperature following fermentation has a significant effect on the pH and viable counts of the BFM preparations (Fig. 1). For samples stored at 4°C, there was no significant change in pH and the number of viable cells declined gradually over a period of 9 weeks. For samples stored at 37°C, the number of viable cells declined more rapidly with no viable cells detected after a period of 7 d. As expected, there was a decrease in pH during storage due to the fermentation of residual sugars. A high pressure treatment of 400 MPa for 5 min (Table 1) and a heat treatment of 72ºC for 1 min were found to be sufficient to kill all detectable viable cells in BFM.

4.2 Determination of culture viability, total cell counts and membrane integrity (LIVE/DEAD staining) of freeze dried powders

The number of culturable cells after freeze-drying was determined as CFU/g. The viable-BFM powders contained approximately 7.3 to 8.5 $\log_{10}$ CFU per gram (Table 2). No culturable cells were detected in dead BFM powders.

Flow cytometric analysis allowed enumeration of total bacterial cells in the freeze dried powders. These counts were used for calculating the bacterial cell: PBMC ratios in

subsequent experiments. Background fluorescence from residual milk particles was easily distinguished from bacterial cells and was excluded from the analysis. The number of total cells present in BFM after freeze-drying was determined as $\log_{10}$ bacteria/g. Total counts ranged from 9.5 to 10.4 $\log_{10}$ bacteria/g (Table 3). Membrane permeability of the *Bifidobacterium* cells in BFM preparations was examined using the LIVE/DEAD stain on the freeze dried powders before and after the lethal treatments (Fig. 2). In general, treatment with either heat or high pressure increased the membrane permeability of the bifidobacteria, allowing access of the propidium iodide component, which increased the number cells which were stained red (DEAD or damaged) and decreased the number of cells which were stained green (LIVE). Storing the BFMs at 37ºC significantly increased the membrane permeability of *B. infantis* 35624 only. On the other hand, storing the BFMs at 4ºC decreased membrane permeability, preventing access of the propidium iodide component which decreased the number of cells which were stained red and increased the number of cells which were stained green. Therefore although virtually all bifidobacteria cells had lost their culturability, a remarkable fraction of the population had retained a functional cell membrane.

4.3 Secretion of cytokines by BFM

All viable-BFMs, the malted milk control and CH2007 induced secretion of IL-10 and TNF-α, albeit to different extents (Fig. 3). The BFMs, with the exception of *B. longum* 0103, stimulated the production of significantly more IL-10 than the milk control (Fig. 3). CH2007 did not induce significantly more IL-10 than the milk control, indicating that the milk itself induces IL-10 in amounts comparable to the lyophilised commercial product. Milk fermented with *B. infantis* 35624 and *B. longum* 0106 appeared to be the most potent IL-10 inducers, provoking on average a 2-3 fold higher IL-10 secretion than the malted milk control. On the other hand, *B. longum* 0103 induced the lowest IL-10 response.

As regards TNF-α, *B. breve* UCC2003 and *B. longum* 0103 and CH2007 induced significantly more TNF-α than the milk control. *B. infantis* 35624 on the other hand induced TNF-α at comparable levels to the milk control. Similarly, *B. longum* 0106 only induced significantly more TNF-α than the milk control at the lowest dilution.

When cytokine profiles were expressed as ratios of IL-10:TNF-α (anti-inflammatory/pro-inflammatory), distinct patterns emerged for the bifidobacterial strains (Fig. 4). At the lowest concentration (10 bacterial cells: PBMC), the fermented milks did not induce a significantly higher ratio of IL-10:TNF-α than the milk control. However, at a higher concentration (25 bacterial cells: PBMC) *B. infantis* 35624 induced a significantly higher ratio of IL-10:TNF-α than the milk control ($P<0.05$), *B. longum* 0103 ($P<0.001$) and CH2007 ($P<0.001$) while *B. longum* 0106 induced a significantly higher ratio of IL-10:TNF-α than *B. longum* 0103 ($P<0.001$), and CH2007 ($P<0.001$). At the highest concentration (50 bacterial cells: PBMC) *B. infantis* 35624 induced a significantly higher ratio of IL-10:TNF-α than *B. breve* UCC2003 ($P<0.01$), *B. longum* 0103 ($P<0.001$) and CH2007 ($P<0.001$).

The IL-10 and TNF-α response in PBMCs (Fig. 5 & 6) was measured after lethal treatment (storage at low pH, heat and high pressure) of the BFM and was also expressed as a ratio of IL-10:TNF-α (Fig. 7 & 8). In general, killing the bacterial cells by storing the BFM at 4°C (ST4-BFM) had no significant impact on the induction of IL-10 or TNF-α (Fig. 5) nor on the ratio of IL-10:TNF-α (Fig. 7). However, storage at 37ºC significantly reduced the induction of IL-10 by *B. breve* UCC2003, compared to viable-BFM, while it significantly increased the induction of TNF-a by *B. longum* 0106. Consequently, storage at 37ºC significantly reduced the ratio of IL10:TNF-α induced by both *B. breve* UCC2003 and *B. longum* 0106. Storage of the malted milk control at 4ºC and 37ºC had no significant impact on the induction of IL-10 or TNF-α in PBMCs

The PBMC responses of heat-treated BFMs (HT-BFM) and high pressure-treated BFMs (HP-BFM) were also compared to the viable-BFM (Fig. 6). For *B. infantis* 35624, the HT-BFM induced a significantly lower IL-10 response while the HP-BFM induced a similar IL-10 and TNF-α response to the viable-BFM. Consequently, heat treatment was found to significantly reduce the ratio of IL-10:TNF-α induced by the *B. infantis* 35624 while high pressure treatment had no significant impact on the ratio (Fig. 8).

For *B. breve* UCC2003, the HT-BFM induced a lower IL-10 response which was significant for only one dilution (25 bacteria: PBMC; $P<0.001$). Similarly, the HP-BFM also induced a lower IL-10 response which was significant for two dilutions (25 bacteria: PBMC; $P<0.01$ and 10 bacteria: PBMC; $P<0.05$). The HP and HT-BFM (*B. breve* UCC2003) both induced comparable levels of TNF-α to the viable

preparation, except for the lowest dilution (10 bacteria: PBMC) which induced significantly more TNF-α than the viable-BFM ($P<0.05$). For *B. longum* 0103, the HT-BFM and HP-BFM induced comparable levels of IL-10 and TNF-α to the viable-BFM, as was the case for *B. longum* 0106. However, the HT-BFM (*B. longum* 0106) induced significantly less IL-10 than the viable-BFM for one dilution (25 bacteria: PBMC; $P<0.05$) and also induced significantly more BFM than the viable-BFM for the lowest dilution (10 bacteria: PBMC; $P<0.001$). Heat-treatment or high pressure-treatment of the malted milk control had no significant impact on the induction of IL-10 or TNF-α in PBMCs.

5.0 Discussion

In this study, the effect of four *Bifidobacterium* strains, grown in fermented milk, on the secretion of the cytokines IL-10 and TNF-α using human PBMCs as an *in vitro* model of host response was investigated. The *Bifidobacterium* strains were shown to induce cytokine production in a strain-specific manner, an observation also made in previous studies (Dong *et al.,* 2011; López *et al.,* 2010; Medina *et al.,* 2007). This strain dependent induction of cytokines highlights the importance of strain identification and characterisation when assessing the immunomodulatory properties of potential probiotic bacteria. The malted milk control also induced cytokine production. Evidence suggests that major components of bovine milk (e.g. milk proteins such as α-casein and lactoferrin) have immunomodulatory effects, including modulation of the cytokine production by activated macrophages (reviewed in: Cross & Gill, 2000).

The BFMs showed a dose-dependent change in their IL-10 and TNF-α stimulating capacity, which has been reported previously (Kekkonen *et al.,* 2008). The stimulation of TNF-α was typically high at the lower doses of bacteria, while the IL-10 secretion increased with bacterial load. The IL-10/TNF- α ratio thus increases with increasing dose. The lowest dose yielded an IL-10/TNF- α ratio below 1, with no significant differences between the strains and the malted milk control. However, at higher doses, *B. infantis* 35624 stimulated a significantly higher IL-10/TNF-α ratio than either the milk control, *B. breve* UCC2003, *B. longum* 0103 or CH2007 (commercially prepared *B. infantis* 35624). As CH2007 is a commercially prepared product (Chr. Hansen), information on the composition of the fermentation medium is unavailable. It is therefore difficult to conclude why this commercial preparation stimulates a

significantly lower anti-inflammatory response compared to the *B. infantis* 35624 BFM. It has previously been shown that the former exerts an anti-inflammatory effect *in vitro* using the PBMC assay (Alimentary Health - data not shown) and also *in vivo* (Konieczna *et al.,* 2012; O'Mahony *et al.,* 2005). Although the present study has shown that *B. infantis* 35624 fermented milk induces a greater anti-inflammatory response than CH2007 *in vitro,* this does not guarantee similar functioning *in vivo*.

It has been noted that although the PBMC model used in this study is not fully reflective of the *in vivo* situation in which intestinal bacteria interact with Peyer's patches, M cells, dendritic cells, macrophages and T cells, it is useful as a screening tool to identify potential immunomodulatory traits of probiotic strains and to select probiotic strains for use in animal models and clinical trials (Dong *et al.,* 2011; Kekkonen *et al.,* 2008). Therefore, the results from the present study may be useful in extrapolating to *in vivo* studies and for making predictions about the potential clinical uses of these probiotic products. This screening project was undertaken with the industry partner, Alimentary Health Ltd., where preliminary work identified IL-10 and TNF-α to be the best representative of an anti- and pro-inflammatory cytokine, respectively, due to their low coefficients of variation (CV) for between-subject variability in the PBMC assay (data not shown). IL-10 and TNF-α play opposing roles in inflammatory responses and their relative balance is of central relevance for regulating immune dysfunction (Dong *et al.,* 2011). IL-10 is an anti-inflammatory cytokine that inhibits antigen presentation and the subsequent release of inflammatory cytokines, thereby controlling inflammatory responses to intestinal antigens and restoring tolerance of T cells to the intestinal microbiota (Lammers *et al.,* 2003; Sanchez-Muñoz *et al.,* 2008). TNF-α, on the other hand, is a pro-inflammatory cytokine involved in the stimulation of inflammatory cytokine production, enhancing the phagocytic/bactericidal properties of macrophages, and activating apoptotic pathways (Schottelius *et al.,* 2004). Moreover, TNF- α has been found to be up-regulated in patients with IBD (Sanchez-Muñoz *et al.,* 2008).

A number of studies have indicated that viable *Bifidobacterium* strains are not always necessary for induction of immunomodulation *in vitro* (Hiramatsu *et al.,* 2007; Hosono *et al.,* 1997). We investigated whether the immunomodulatory effects observed during treatment with BFM could be maintained even after the *Bifidobacterium* cells had been rendered non-culturable by different lethal treatments. The methods employed here to

kill bacteria included heat treatment, high pressure treatment and storage at low pH. The heat treatment method used to kill the bacteria has been used previously where researchers have investigated its immunomodulatory effects both *in vitro* and *in vivo* (Cross *et al.*, 2004; Hiramatsu *et al.*, 2007; Li *et al.*, 2009; Matsuguchi *et al.*, 2003; Xiao *et al.*, 2002; Zhang *et al.*, 2005). However, to the best of our knowledge, high pressure treatment and storage at low pH have not been used as methods for killing bacteria when they are screened for their immunomodulatory properties either *in vitro* or *in vivo*.

The effect of storage at a low pH on the *Bifidobacterium* cells was strongly dependent on the storage temperature. Storage of the BFM at 37°C resulted in a rapid decline of culturable cells, whereas storage at 4°C resulted in a more gradual decline. This rapid decline in culturable cells at the higher storage temperature can be explained, to some extent, by the additional decrease in pH (from approximately 4.6 to 4.0). Indeed, it has been found that most strains of bifidobacteria are sensitive to pH values below 4.6 (Boylston *et al.*, 2004; Modler, 1994) and similar to the present study, Modler (1994) found that a bifidobacterial population could decline from 10^9 to 0 in less than a week in a low pH yoghurt (pH 3.9–4.0). Staining with the fluorescent probes (SYTO-9 and propidium iodide (PI)) and subsequent analysis by flow cytometry revealed that storage at 37ºC increased the membrane permeability of bacterial cells while storage at 4ºC decreased membrane permeability, the reasons for which are unknown. It must be noted that the SYTO-9 and PI dyes can bind components from the medium in unpredictable ways, resulting in variation in staining efficiency between samples. Hence these results should be interpreted with caution. Nonetheless, it has previously been reported that *Bifidobacterium* cells (in a fermented oat drink) lose culturability when stored at 4ºC while maintaining an intact membrane (Lahtinen *et al.*, 2006). Storage at low pH also had varying effects on the immunomodulatory capacity of the BFM depending on strain and storage temperature. In general, killing the cells by storage at 4ºC had no significant impact on the induction of IL-10 or TNF-α. However, storage at 37ºC reduced the IL-10 signal induced by *B. breve* UCC2003 and increased the TNF-α signal induced by *B. longum* 0106.

High pressure treatment was also used as a method to kill *Bifidobacterium* cells in the fermentates. In HP processing, food is subjected to pressures usually within the range of

300-700MPa and values of around 400MPa are usually effective in killing most of the vegetative bacteria (Chawla *et al.,* 2011). The effects of pressure on microorganisms in food are determined by the temperature during pressure treatment, the food constituents and the physiological state of the microorganism (Chawla *et al.,* 2011). Tsevdou & Taoukis (2011) recently assessed the effect of high hydrostatic pressure on the survival of *Bifidobacterium* spp. in broth and found that high pressure treatments of 200MPa at 20-15°C for 10-15 min were not detrimental to the viability of the culture (Tsevdou and Taoukis, 2011). Consequently, we examined the effect of high pressure treatments >300MPa on viability of our strains in a milk product and a treatment of 400MPa for 5 min was found to be sufficient to kill all culturable cells and increased membrane permeabilisation. Indeed, damage to cell membranes, is one of the main causes of death of microorganisms by high pressure treatment (Lado and Yousef, 2002). For *B. infantis* 35624, *B. longum* 0106 and *B. longum* 0103 the high pressure treated BFM samples induced comparable IL-10 and TNF-α responses to the viable BFM. However, for *B. breve* UCC2003, high pressure treatment significantly decreased the induced IL-10 signal and increased the TNF-α signal.

Heat treatment of BFM in a water bath resulted in complete loss of viable cells and an increase in membrane permeability. Heat treatment of the *B. infantis* 35624–fermented milk samples resulted in a significant decrease in the levels of IL-10 induced in PBMCs. This may be explained by the impact that heat treatment has on cellular integrity and protein structure (Lopez *et al.,* 2008). However, heat treated BFM induced comparable levels of TNF-α to the viable form. Similarly, for *B. breve* UCC2003 and *B. longum* 0106, the HT-BFM induced a significantly lower IL-10 response and a higher TNF-α response compared to the viable BFM. However, for *B. longum* 0103, heat treatment had no affect on its ability to induce the cytokines, IL-10 and TNF-α.

It has been reported previously that live bacteria are more potent inducers of cytokine production in mammalian leucocytes than killed forms (Cross *et al.,* 2004; Miettinen *et al.,* 1996). Cross *et al*. (2004) demonstrated that live *L. casei* Shirota induced a murine monocytes/macrophage cell line to secrete higher levels of IL-12 and TNF-α than those observed using a comparable number of heat killed bacilli. Similarly, Miettinen *et al*. (1996) demonstrated that several live lactic acid bacteria (LAB), but not gluteraldehyde-fixed LAB, were potent inducers of TNF-α release from human PBMCs. However, in

contrast, a number of other studies have shown that the live condition is not necessary for bacteria to exert an immune response (Hiramatsu *et al.,* 2007; Lammers *et al.,* 2003). Hiramatsu *et al.* (2007) found that live, ultra-sonic treated and heat treated *Bifidobacterium* co-cultured with murine Peyer's patch cells induced IL-12p40 *in vitro.* In fact, the sonicated *Bifidobacterium* induced significantly more IL-12p40 than the live form in the murine Peyer's patch cells. The authors concluded that disruption of bacterial cell walls following ultra-sonic treatment led to increased exposure of microbe-associated molecular patterns (MAMPS) such as peptidoglycans, lipoteichoic acids and DNA. Indeed, Lammers *et al.* (2003) have previously found that *Bifidobacterium* genomic DNA induced secretion of IL-10 by human PBMC.

The present study showed that the stimulatory activity of the *Bifidobacterium*-fermented milk differed, depending on the particular strains, the concentrations and the treatments applied to render the bifidobacteria non-culturable. Of all of the methods employed in this study to render the bacterial cells non-culturable, storage at 4ºC appeared to be the best method for maintaining immunomodulatory activity in all *Bifidobacterium* strains. Storage at 37ºC resulted in a reduced anti-inflammatory signal, particularly in *B. breve* UCC2003 and *B. longum* 0106. Heat treatment reduced the anti-inflammatory signal of *B. infantis* 35624 and *B. breve* UCC2003 while high pressure treatment reduced the anti-inflammatory signal of *B. longum* 0103. Interestingly, the induction of both IL-10 and TNF-α by the malted milk control was largely unchanged irrespective of the treatment used, indicating that the different treatments did not significantly affect the immunomodulatory capacity of the malted milk components.

It would be interesting to know whether the strain specific cytokine release demonstrated in this study is related to EPS production of each strain. A number of studies have demonstrated the immunomodulatory capacity of EPS (Arena *et al.,* 2006; Ciszek-Lenda *et al.,* 2011; Hidalgo-Cantabrana *et al.,* 2012; López *et al.,* 2012; Wu *et al.,* 2010). However, the quantity of EPS produced in the BFM has not yet been determined and thus it is not possible to predict its effects. The only useful information available is the appearance of bifidobacteria colonies on Congo red agar. Indeed, *B. infantis* 35624 and *B. longum* 0106, which induced a higher IL-10/TNF-α ratio than *B. breve* UCC2003 and *B. longum* 0103, form whiter colonies which exhibit a more mucoid appearance on Congo red agar (data not shown). Nonetheless, several other

components produced from milk fermentation also may contribute to the immunomodulatory activity of the BFMs. It has been shown that supernatants of milk fermented by *L. helveticus* modulated the *in vitro* proliferation of lymphocytes by acting on cytokine production (Laffineur *et al.*, 1996). In addition, cell-free extracts of the fermented milk product kefir were able to induce the *in vivo* production of cytokines by peritoneal macrophages and adherent cells isolated from Peyer's patches (Vinderola *et al.*, 2006).

The intestine is a complex ecosystem in which the microbiota and the host interact in order to uphold an immunologically balanced intestinal inflammatory response (Dicksved and Willing, 2011; Lammers *et al.*, 2003; Tamboli *et al.*, 2004). Investigation into the mechanisms by which intestinal microorganisms, including 'non-viable' probiotics, regulate the immune response may allow formulation of probiotic supplements that are tailored for particular disorders based on their immunomodulatory properties (López *et al.*, 2010). In this regard, characterising how the immune system responds to probiotic bacteria *in vitro* may give an indication as to the likely immunomodulatory events that can be triggered following probiotic administration *in vivo*. In general, BFMs containing dead cells stimulated levels of IL-10 and TNF-α which were comparable to those stimulated by BFM containing live cells. Taking into account that the manufacturing process and gastrointestinal passage affect bacterial viability, these results obtained in this study may indicate a potential therapeutic effect of non-viable bacteria ingested in BFM. Moreover, as well as eliminating shelf life problems, administration of non-viable microorganisms could reduce the risk of microbial translocation and infection, particularly in individuals with compromised gut barrier function. While the results have clearly shown that microbiologically inactive *Bifidobacterium* strains are capable of inducing a cytokine response upon direct interactions with PBMCs, the true *in vivo* picture needs to be fully determined taking into account the contributing roles of other commensal gut bacteria as well as the immunocompetent cells in the gut environment. However, this production of a microbiologically non-viable yet immunologically active probiotic product may have applications in human health and would have a noticeable advantage compared to other 'viable' probiotics by allowing for the generation of safer more stable products (e.g. longer shelf life).

Table 1. Inactivation of *Bifidobacterium* strains in a malted milk preparation by application of HP. Results are expressed as the mean (± standard error) of triplicate independent experiments

Pressure Treatment	**Log_{10} CFU mL^{-1}**			
	B. infantis 35624	*B. breve* UCC2003	*B. longum* 0103	*B. longum* 0106
Atm x 5 min	8.76 ± 0.05	9.29 ± 0.02	8.92 ± 0.04	8.81 ± 0.08
300 MPa	1.50 ± 0.62	2.05 ± 0.22	1.22 ± 0.27	1.24 ± 0.40
400 MPa	<1.00	<1.00	<1.00	<1.00
500 MPa	<1.00	<1.00	<1.00	<1.00
600 MPa	<1.00	<1.00	<1.00	<1.00

Table 2. Culture viability of freeze-dried BFMs [Untreated BFM (Viable-BFM), BFM stored at 37°C (ST37-BFM), BFM stored at 4°C (ST4-BFM), heat-treated BFM (HT-BFM) and high pressure-treated BFM (HP-BFM). Results are expressed as the mean (± standard error) of triplicate independent experiments

	Log_{10} CFU g^{-1}					
Strain	**viable-BFM**	**ST37-BFM**	**ST4-BFM**	**viable-BFM**	**HT-BFM**	**HP-BFM**
B. infantis 35624	7.72 ± 0.30	<1.00	<1.00	7.32 ± 0.19	<1.00	<1.00
B. breve UCC2003	7.99 ± 0.01	<1.00	<1.00	8.47 ± 0.01	<1.00	<1.00
B. longum 0103	8.08 ± 0.24	<1.00	<1.00	7.95 ± 0.11	<1.00	<1.00
B. longum 0106	8.19 ± 0.10	<1.00	<1.00	8.10 ± 0.0	<1.00	<1.00

Table 3. Total cell counts of freeze-dried BFMs [Untreated BFM (viable-BFM), BFM stored at 37°C (ST37-BFM), BFM stored at 4°C (ST4-BFM), heat-treated BFM (HT-BFM) and high pressure-treated BFM (HP-BFM)] enumerated using flow cytometry. Results are expressed as the mean (± standard error) of triplicate independent experiments.

	Log_{10} bacteria g^{-1}					
Strain	**viable-BFM**	**ST37-BFM**	**ST4-BFM**	**viable-BFM**	**HT-BFM**	**HP-BFM**
B. infantis 35624	10.03 ± 0.08	10.09 ± 0.09	10.23 ± 0.13	9.92 ± 0.02	9.81 ± 0.09	9.80 ± 0.11
B. breve UCC2003	9.89 ± 0.08	10.10 ± 0.05	10.30 ± 0.11	10.35 ± 0.08	10.04 ± 0.10	9.54 ± 0.13
B. longum 0103	9.80 ± 0.13	9.87 ± 0.06	10.24 ± 0.08	9.65 ± 0.08	9.52 ± 0.04	9.47 ± 0.06
B. longum 0106	9.95 ± 0.14	9.69 ± 0.07	10.09 ± 0.09	10.09 ± 0.16	9.76 ± 0.10	9.93 ± 0.01

Figure 1. Changes in viable counts and pH of *Bifidobacterium* strains in fermented milk samples during storage at (A) 37°C and (B) 4°C: *B. infantis* 35624, *B. breve* UCC2003, *B. longum* 0103 and *B. longum* 0106. Results are expressed as the mean (± standard error) of triplicate independent experiments

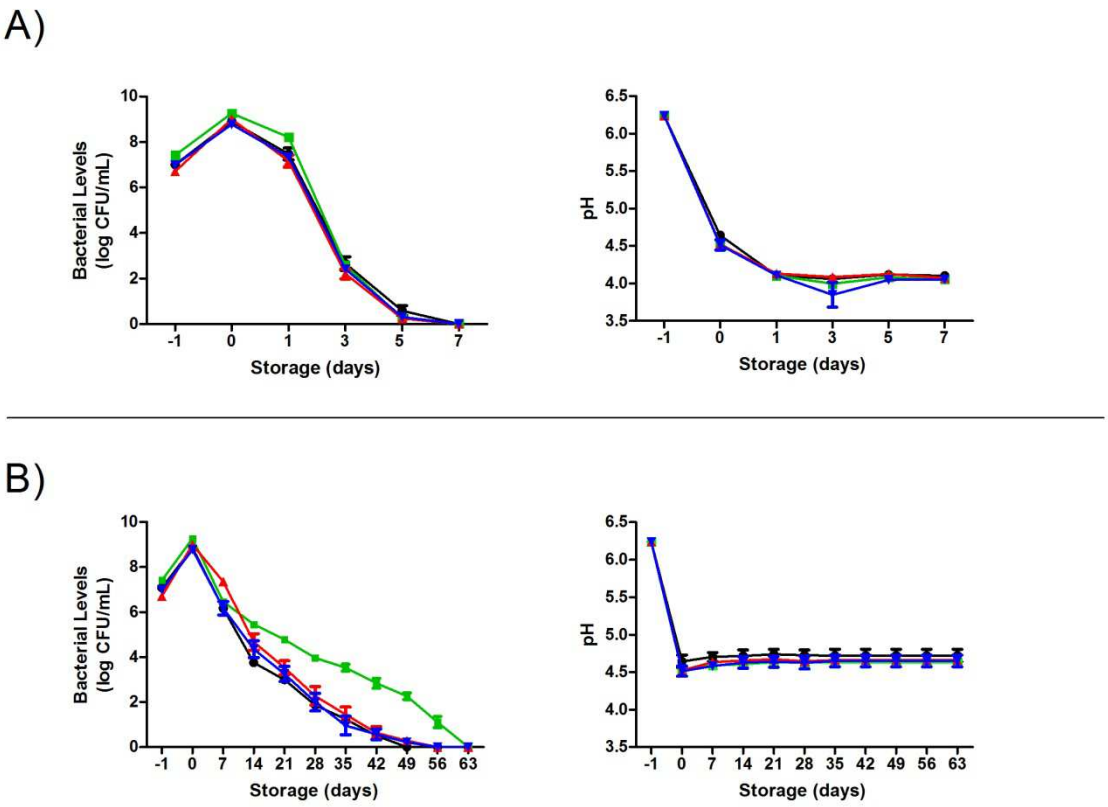


Figure 2. Percent permeability of freeze-dried BFM samples using LIVE/DEAD staining and flow cytometry (■ *B. infantis* 35624, ■ *B. breve* UCC2003, ■ *B. longum* 0103, and ■ *B. longum* 0106). Results are expressed as the mean (± standard error) of 3 independent assays. **A)** Untreated BFM (viable-BFM) is indicated with filled bars, BFM stored at 37°C (ST37-BFM) with open bars and BFM stored at 4°C (ST4-BFM) with hatched bars. **B)** Untreated BFM (viable-BFM) is indicated with filled bars, heat-treated BFM (HT-BFM) with open bars and high pressure-treated BFM (HP-BFM) with hatched bars.

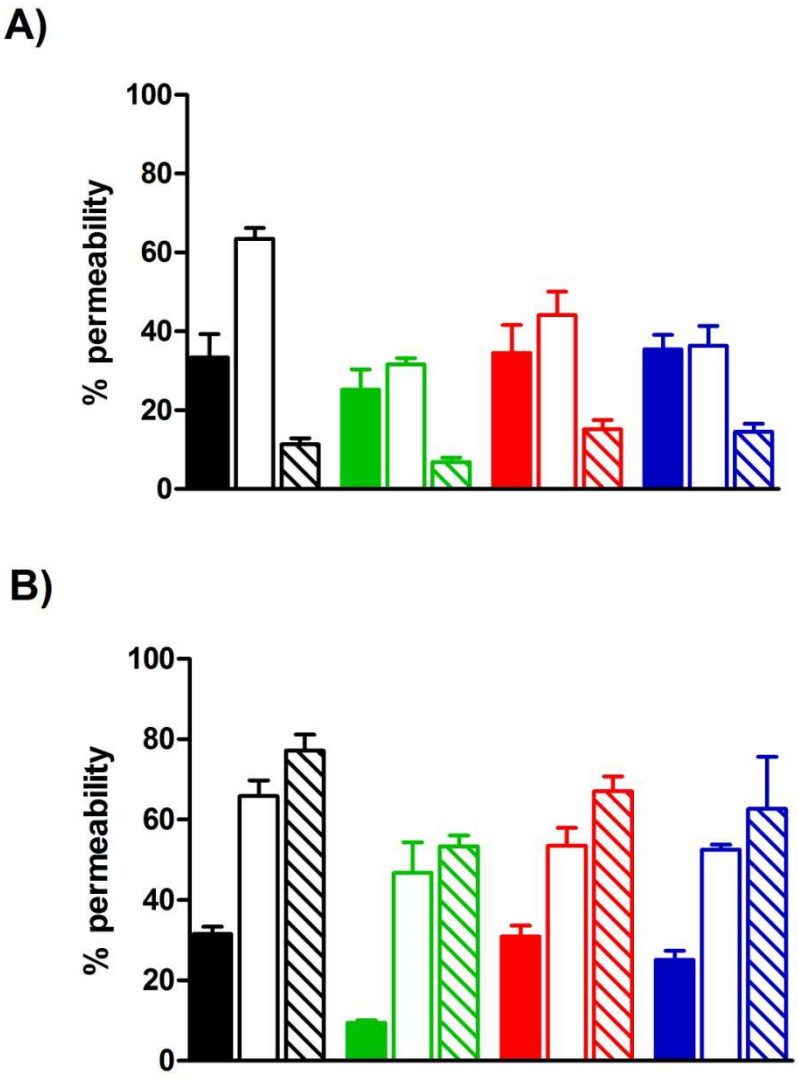


Figure 3. IL-10 and TNF-α production by PBMCs induced by untreated (viable) BFM (■ *B. infantis* 35624, ■ *B. breve* UCC2003, ■ *B. longum* 0103, and ■ *B. longum* 0106), ■ milk control and ■ CH2007 after 48 h stimulation. Results are expressed as the mean (± standard error) of 3 independent assays, each done with PBMC's from 3 different donors. P<0.05 (*) P<0.01 (**), P<0.001 (***) for BFM versus milk control as determined by two-way ANOVA with a Bonferonni correction.

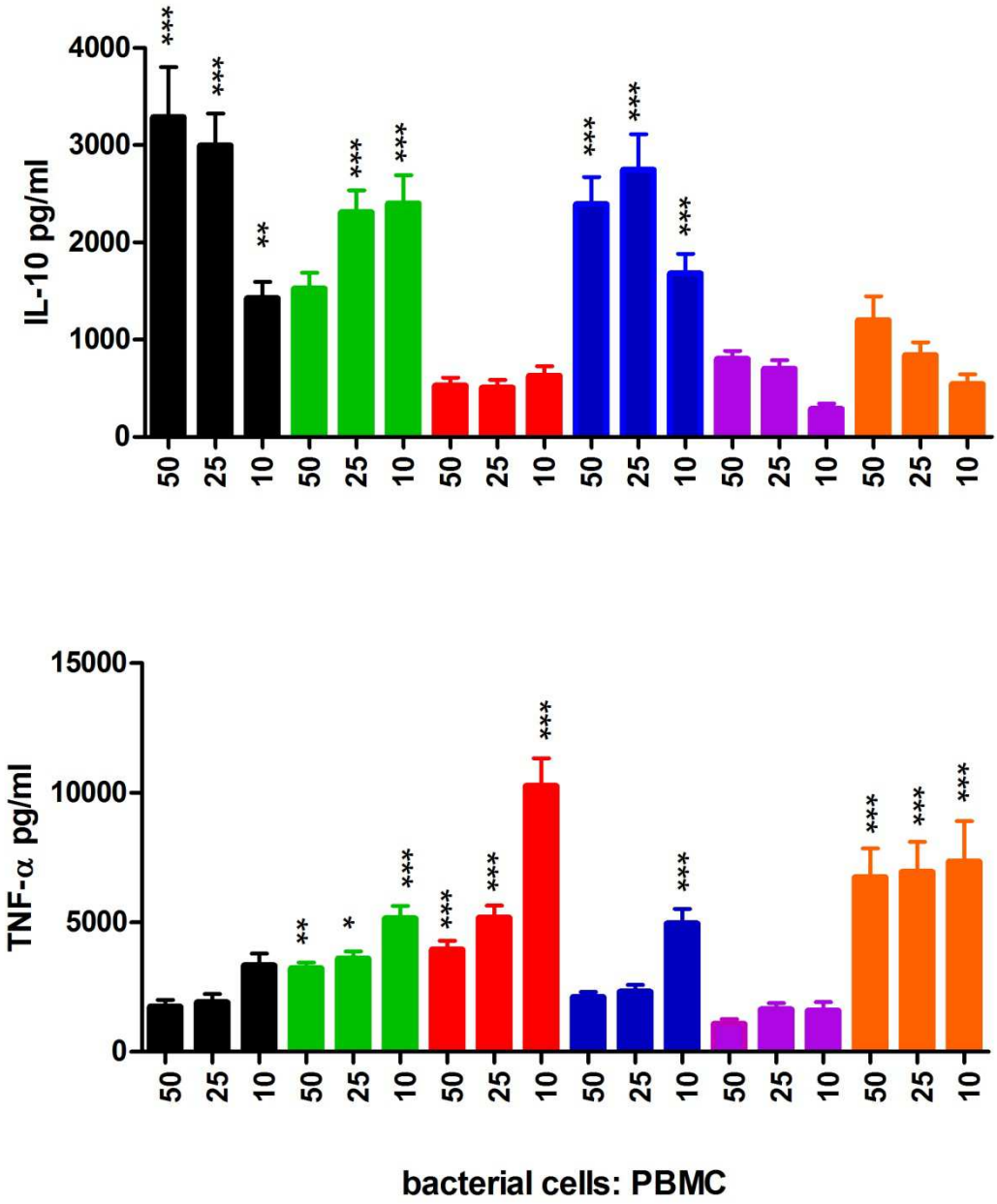


Figure 4. Effect of untreated (viable) BFM (■ *B. infantis* 35624, ■ *B. breve* UCC2003, ■ *B. longum* 0103, and ■ *B. longum* 0106), ■ milk control and ■ CH2007 on ratios of IL-10:TNF-α. Results are expressed as median and interquartile range (whiskers: min to max). P<0.05 (*), P<0.001 (***) for BFM/CH2007 versus milk control as determined by Kruskal Wallis test with a Dunn correction.

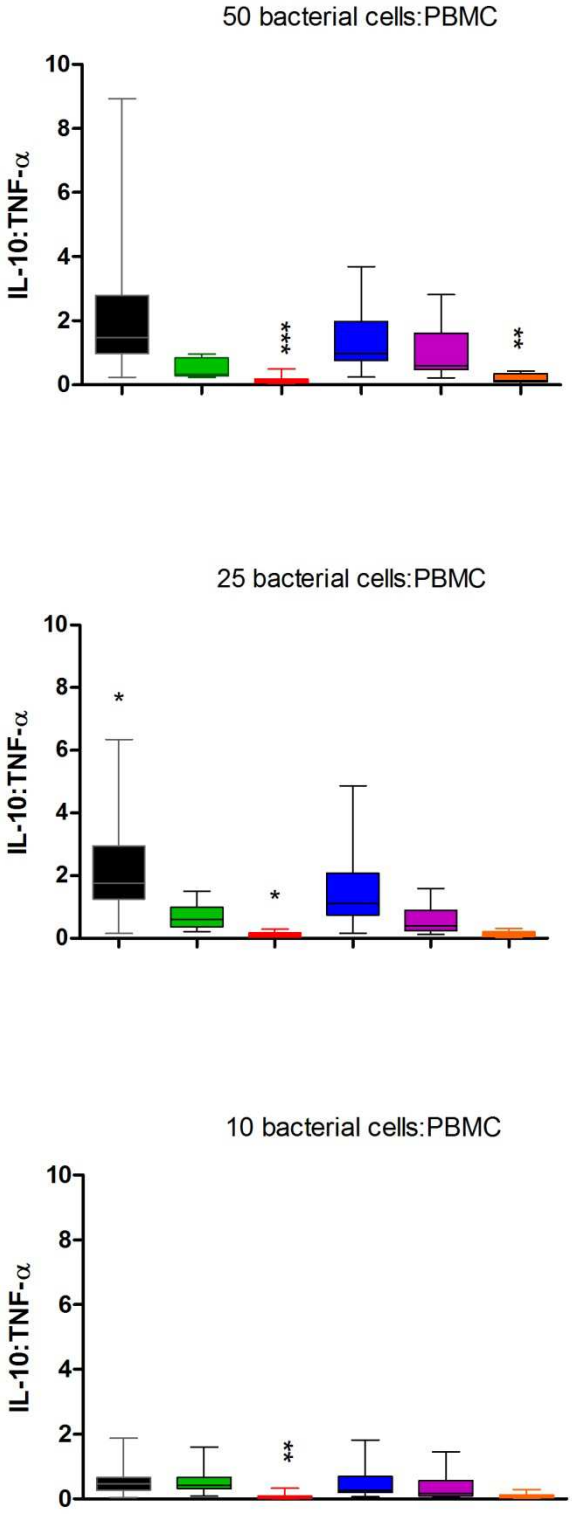


Figure 5. IL-10 and TNF-α production by PBMCs induced by untreated (viable) or treated (non-viable) BFM (■ *B. infantis* 35624, ■ *B. breve* UCC2003, ■ *B. longum* 0103, and ■ *B. longum* 0106) and ■ milk control after 48 h stimulation. Results are expressed as the mean (± standard error) of 3 independent assays. P<0.05 (*) P<0.01 (**), P<0.001 (***) for treated BFM versus untreated BFM as determined by two-way ANOVA with a Bonferonni correction. Untreated BFM (viable-BFM) is indicated with filled bars, BFM stored at 37°C (ST37-BFM) with open bars and BFM stored at 4°C (ST4-BFM) with hatched bars.

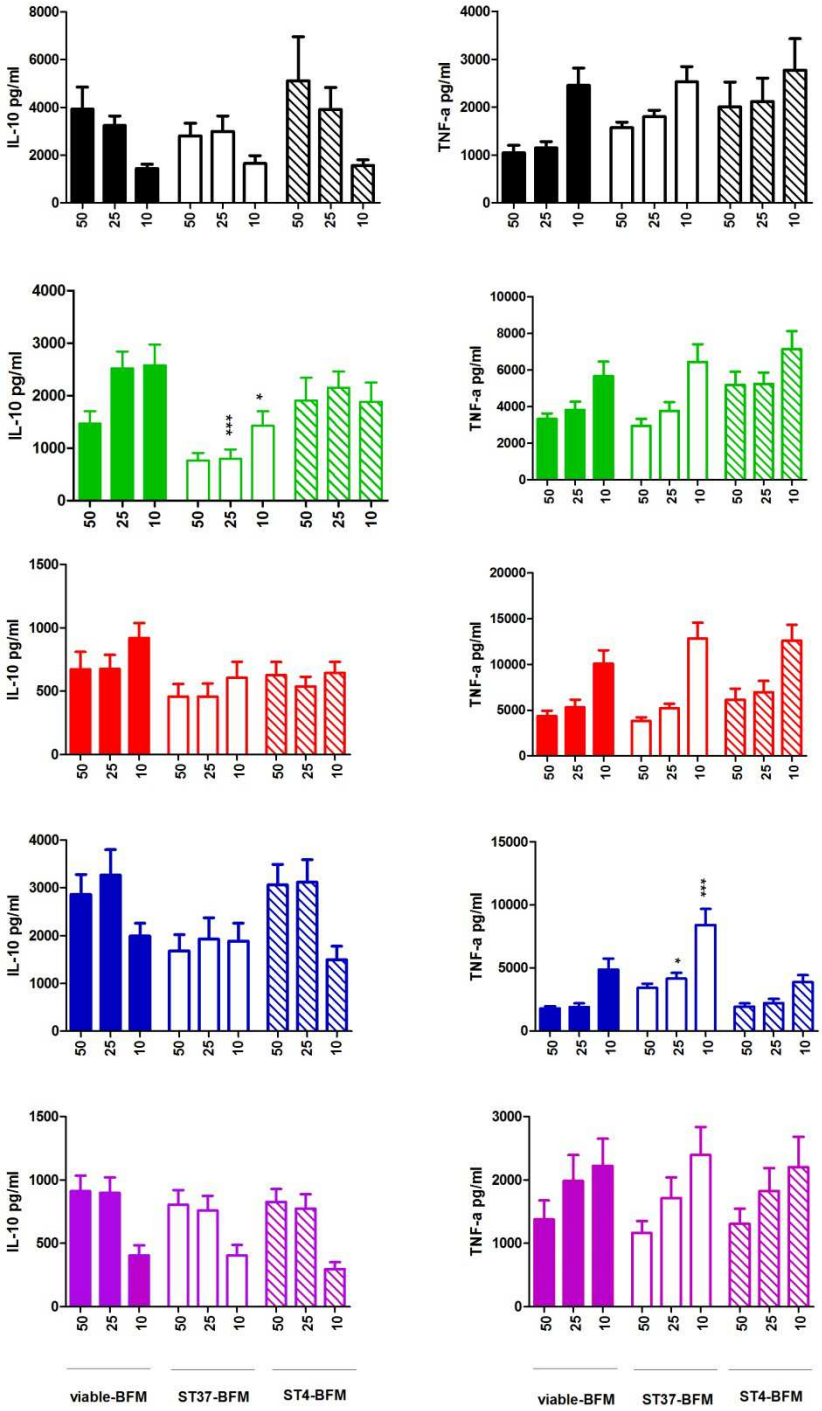


Figure 6. IL-10 and TNF-α production by PBMCs induced by viable or non-viable BFM (■ *B. infantis* 35624, ■ *B. breve* UCC2003, ■ *B. longum* 0103, and ■ *B. longum* 0106) and ■ malted milk control (no bacterial cells) after 48 h stimulation. Results are expressed as the mean (± standard error) of 3 independent assays. Untreated BFM (viable-BFM) is indicated with filled bars, heat-treated BFM (HT-BFM) with open bars and high pressure-treated BFM (HP-BFM) with hatched bars. P<0.05 (*) P<0.01 (**), P<0.001 (***) for viable BFM versus non-viable BFM (HT-BFM or HP-BFM) as determined by two-way ANOVA with a Bonferonni correction

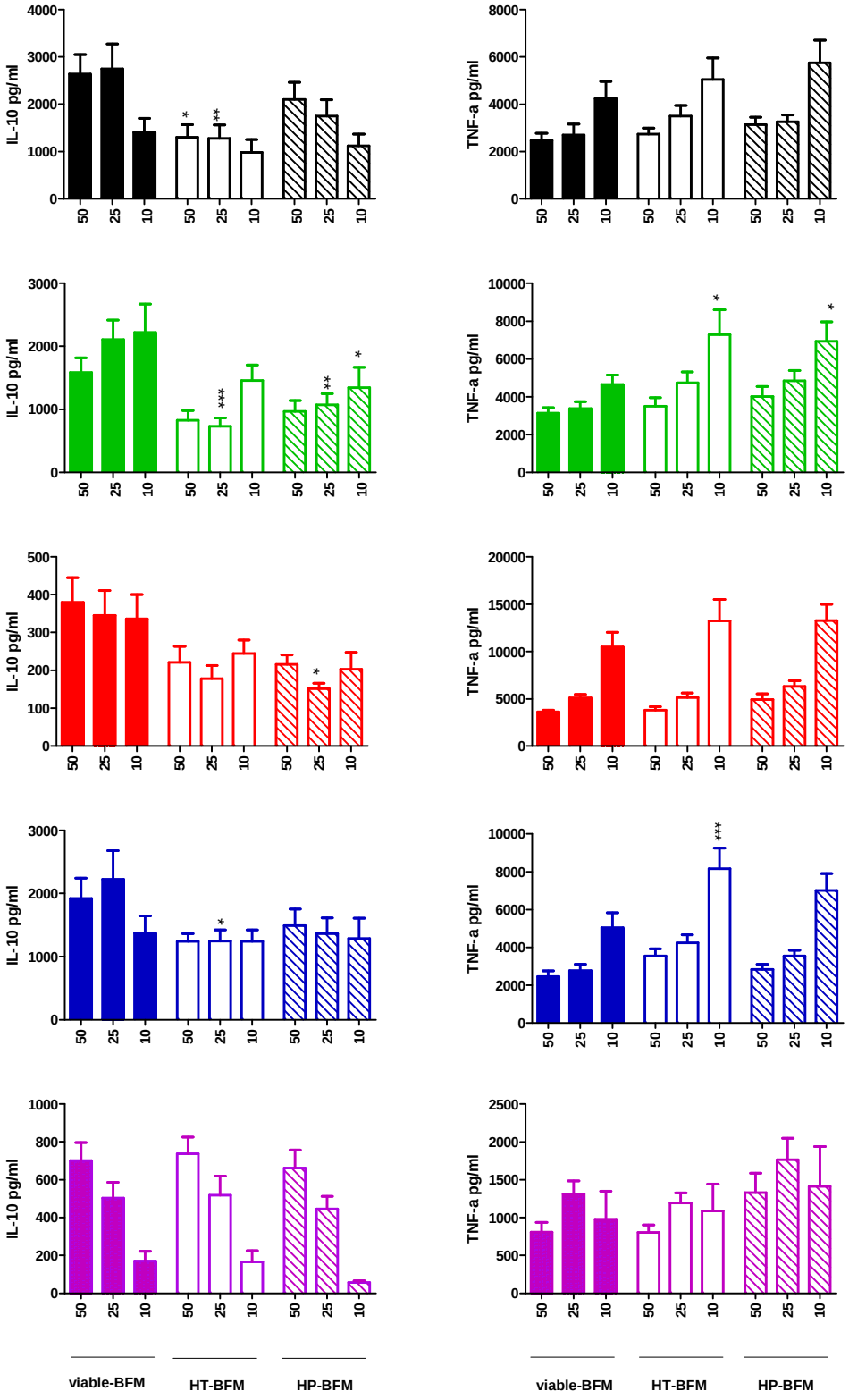


Figure 7. Effect of untreated (viable) BFM and treated (non-viable) BFMs (BFM stored at 37°C and BFM stored at 4°C) on ratios of IL-10:TNF-α (■ *B. infantis* 35624, ■ *B. breve* UCC2003, ■ *B. longum* 0103, ■ *B. longum* 0106) and, ■ milk control). Results are expressed as median and interquartile range (whiskers: min to max).. P<0.05 (*) P<0.01 (**), P<0.001 (***) for treated BFM versus untreated BFMs (of same dilution) as determined by Kruskal Wallis test with a Dunn correction. Untreated BFM (viable-BFM) is indicated with filled bars, BFM stored at 37°C (ST37-BFM) with open bars and BFM stored at 4°C (ST4-BFM) with hatched bars.

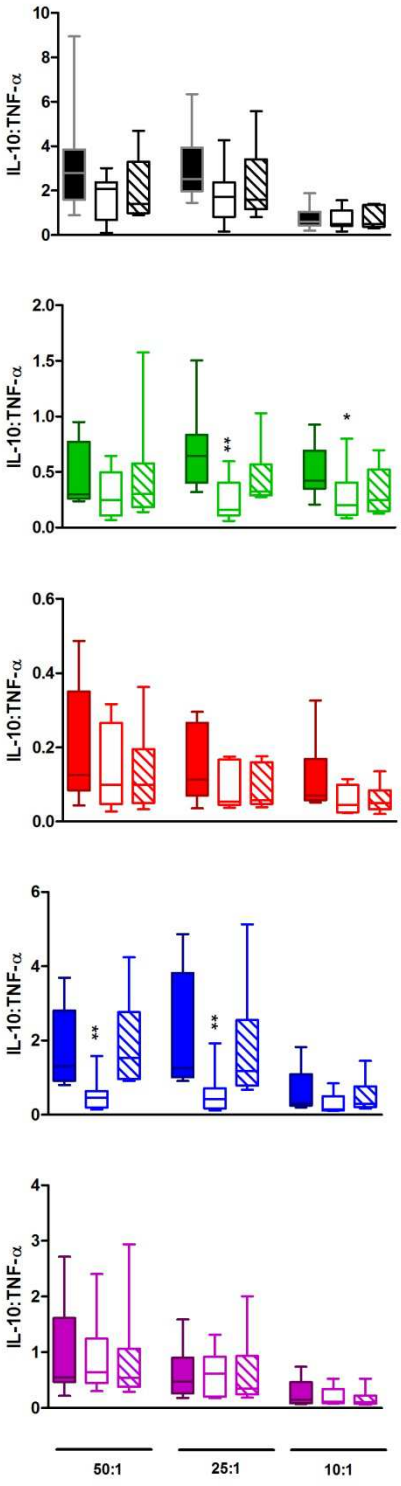
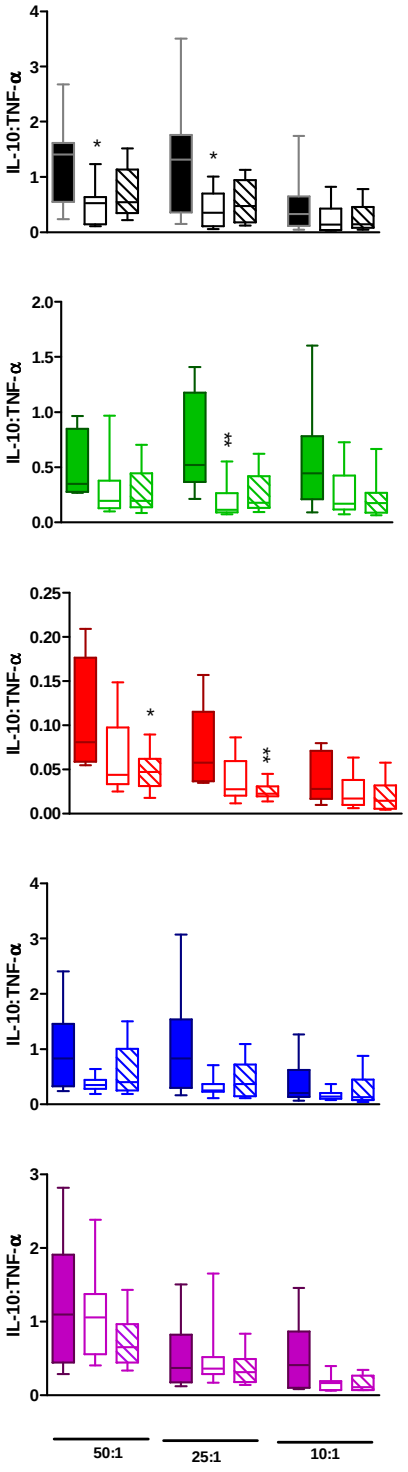


Figure 8. Effect of untreated (viable) BFM and untreated (non-viable) BFMs (heat-treated BFM and high pressure treated BFM) on ratios of IL-10:TNF-α (■ *B. infantis* 35624, ■ *B. breve* UCC2003, ■ *B. longum* 0103, ■ *B. longum* 0106) and, ■ milk control). Results are expressed as median and interquartile range (whiskers: min to max). P<0.05 (*) P<0.01 (**), P<0.001 (***) for treated BFM versus untreated BFMs (of same dilution) as determined by Kruskal Wallis test with a Dunn correction. Untreated BFM (viable-BFM) is indicated with filled bars, heat treated BFM (HT-BFM) with open bars and high pressure treated BFM (HP-BFM) with hatched bars.



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CHAPTER 6

Antimicrobial production by intestinal bacteria from elderly subjects

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1.0 Abstract

The human gastrointestinal tract (GIT) harbours a complex microbial community which plays an intricate role in health and disease. Many species that make up the intestinal microbiota produce antimicrobial compounds which may confer a competitive advantage to producing organisms over other intestinal isolates, possibly facilitating dominance and aiding survival within the intestine. The objective of this study was to investigate the prevalence of antimicrobial production in the human gut microbiota and to mine this rich repository of metabolites for therapeutic potential. Faecal samples, obtained from 42 elderly subjects (≥65 yrs) and 7 non-elderly volunteers (22-57 yrs), were plated on to different growth media for the isolation of 1) total anaerobes; 2) non-spore forming anaerobes; 3) Gram negative anaerobes; 4) total *Enterobacteriaceae;* and 5) *Bacteroides.* Selected colonies from each plate were screened for inhibitory substances against four target organisms, *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901, *Escherichia coli* K-12, *Bifidobacterium breve* UCC2003, and *Cronobacter sakazakii* DPC6440. The data suggest that antimicrobial production by intestinal isolates against our chosen targets under the screening conditions used was not frequent (1.1% for *E.coli* K-12 and 0.2% for *Lb. bulgaricus* LMG6901). No isolates were found to inhibit *B. breve* UCC2003 and *C. sakazakii* DPC6440. Sixteen genetically distinct strains producing antimicrobial substances were investigated. A number of previously characterised bacteriocins including ABP-118 (from *Lactobacillus salivarius),* gassericin T *(Lactobacillus gasseri),* mutacin II *(Streptococcus mutans)* and enterocin L-50 and enterocin P (*Enterococcus faecium)* were identified. A number of *E. coli* strains were also isolated which produced heat labile antimicrobials, most likely colicins or microcins. Interestingly, a *Lactobacillus crispatus* strain was isolated and was found to produce a potentially novel heat labile antimicrobial compound which was resistant to protease activity in the cell-free supernatant. In conclusion, this extensive screening of the elderly faecal microbiota indicates that the frequency of isolating potentially new antimicrobial compounds is low.

2.0 Introduction

It is estimated that the total microbial load of the human adult gastrointestinal tract (GIT) comprises 10^{13}–10^{14} microbial cells (Gill *et al.*, 2006), outnumbering human somatic and germ cells by a factor of ten (Quigley, 2010). The majority of bacteria are present in the large intestine where over 1000 different species are thought to reside (Ley *et al.,* 2006; Qin *et al.,* 2010). Here, they reach densities of 10^{11}–10^{12} cells/mL of luminal content (Turroni *et al.,* 2008).

Microbes produce an array of antimicrobial compounds which include broad spectrum classical antibiotics, metabolic by-products such as lactic acid, lytic enzymes, several types of protein exotoxins, and bacteriocins (Riley & Wertz, 2002). Bacteriocins are defined as ribosomally synthesised antimicrobial peptides produced by numerous bacteria which exhibit inhibitory activity against bacteria, either in the same species (narrow spectrum) or across genera (broad spectrum) (Cotter *et al.,* 2005). Producer organisms are immune to their own bacteriocin(s), a property that is mediated by specific immunity proteins (Cotter *et al.,* 2005).

Bacteriocins were first identified by Gratia in 1925 when a heat-labile substance in *E. coli* V culture supernatant was found to be toxic to *Escherichia coli* S (Gratia, 1925). Since then, hundreds of bacteriocins have been identified among most families of bacteria. It has been suggested that the vast majority of all bacteria and archaea probably produce at least one bacteriocin (Klaenhammer, 1988) which highlights the fundamental role that bacteriocins play in microbial systems (Gillor *et al.,* 2009). However, their precise ecological role is an area of ongoing debate (Dobson *et al.,* 2012)

Bacteriocin production is thought to confer a competitive advantage on the producing strain, possibly allowing it to influence or to dominate complex microbial populations (O'Shea *et al.,* 2009; Ryan *et al.,* 1996; Ryan *et al.,* 2001) such as those in the intestine. In fact, numerous bacteriocins from both Gram positive and Gram negative species resident within the large intestine have been isolated (Gordon & O'Brien, 2006; O'Shea *et al.,* 2009; Rea *et al.,* 2010). Indeed, it has been suggested that colicin production plays a significant role in the colonisation of *E. coli* in the GIT. Gillor *et al.* (2009) demonstrated that colicinogenic *E. coli* are able to persist in the large intestine of

streptomycin-treated mice for an extended period of time relative to their non-colicin producing counterparts. Similarly, bacteriocin production by the intestinal isolate *Bifidobacterium longum* subsp. *longum* DJ010A may be an important adaptation for GI survival. Simulated faecal competition studies revealed that *B. longum* DJ010A had a significantly increased ability to compete against *Clostridium difficile* and *E. coli* than did the non-producing isogenic variant *B. longum* DJO10-JH1. However, *in vivo* studies in an intestinal model would be necessary to verify this hypothesis (Lee *et al.,* 2008).

Bacteriocin production may be particularly important as killing peptides *in vivo* and a number of bacteriocins have shown potential for use in the control of gastrointestinal pathogens. *Lactobacillus salivarius* UCC118 produces a bacteriocin *in vivo* that was found to provide significant protection to mice against infection with *Listeria monocytogenes* (Corr *et al.,* 2007). The faecal isolates, *Lactococcus lactis* MM19 and *Pediococcus acidilactic* MM33, producing the bacteriocins nisin Z and pediocin PA-1/AcH, respectively were found to reduce intestinal colonization by vancomycin-resistant enterococci (VRE) in a mouse model (Millette *et al.,* 2008). Moreover, the relatively narrow killing spectrum of bacteriocins (Riley & Wertz, 2002) may reduce the collateral impact on the commensal flora typically seen with administration of antibiotics. A recent study reported that a narrow-spectrum bacteriocin, Thuricin CD, exhibited inhibitory activity against *C. difficile* (in an *ex vivo* model of the colon) which was as effective as conventional antibiotics (i.e. metronidazole and vancomycin) (Rea *et al.,* 2010). Screening the intestinal microbial community may therefore be a practical approach for the isolation of novel bacteriocin producers which may have potential use as alternatives to antibiotics or as probiotics in the population.

The present was based on a two-fold hypothesis: 1) that extensive screening would yield new antimicrobials with potential applications and; 2) that the extent of antimicrobial production would indicate that this feature must be a significant player in determining the balance of the gut microbiota. Hence, the objective of this study was to isolate culturable species from human faecal samples (collected from elderly subjects participating in the ELDERMET study and a group of non-elderly volunteers) and to investigate antimicrobial production by these isolates *in vitro*.

3.0 Materials and Methods

3.1 Faecal sampling, storage and transport to laboratory

Faecal samples were obtained from 42 elderly subjects (≥65 yrs) participating in the ELDERMET study. The subjects included patients from rehabilitation centres (*n* 4), healthy community-dwelling individuals (*n* 19), community-dwelling individuals taking antibiotics (*n* 17) and individuals hospitalised with *C. difficile*-associated diarrhoea (*n* 2) (Table 1). Faecal samples were also obtained from 7 non-elderly volunteers (5 males and 2 females, 22-57 yrs, mean age 33 yrs) who had not taken an antibiotic or probiotic in the previous 3 months.

All faecal samples were collected into sterile containers and stored at 4ºC. ELDERMET samples were delivered to the ELDERMET clinics (Cork University Hospital/St. Finbarr's Hospital, Cork). Upon delivery, approximately 4g of each faecal sample was immediately transferred to a sterile faecal specimen container (only fresh samples < 24 h old) and stored anaerobically in AnaeroGen W-Zip COMPACT pouches (Oxoid Ltd, Basingstoke, Hampshire, UK) at 4^{o}C prior to dispatching on ice to the laboratory. Faecal samples from the non-elderly volunteers were delivered directly to the laboratory and stored anaerobically in Oxoid jars containing Anaerocult A (Merck KGaA, Darmstadt, Germany) as a reducing agent at 4^{o}C. All samples were analysed within 24 h of arrival at the laboratory.

3.2 Isolation and enumeration of anaerobic bacteria

One gram of faecal sample was diluted ten-fold with 50 mM potassium phosphate buffer (pH 7) supplemented with 0.05% (w/v) cysteine hydrochloride (Sigma, Poole, Dorset, U.K) in a Seward stomacher bag. The sample was mixed well in the buffer, filtered to remove any large particles and debris, and transferred immediately to an anaerobic chamber containing an anoxic atmosphere (10% H_2, 10% CO_2, 80% N_2). The homogenised faecal samples were then serially diluted.

Dilutions were spread-plated (100 µL) onto 5 different growth media (Table 2) and incubated anaerobically at 37°C. Antibiotic stocks were dissolved in distilled water and filter sterilized (0.45µM). Plates were examined after 1-3 days. If growth was sufficient,

colonies were enumerated. Bacterial counts were recorded as colony forming units (CFU) per gram of faeces.

3.3 Screening anaerobic faecal isolates for anti-microbial production

3.3.1 Bacterial strains and growth media

All strains were maintained as 40% (v/v) glycerol stock at -80°C. Lactic acid bacteria (LAB) strains were grown anaerobically at 37ºC on de Man, Rogosa, and Sharpe (MRS; Difco Laboratories, Detroit, MI, USA) medium supplemented with 0.05% (w/v) cysteine hydrochloride (Sigma Chemical Co., St. Louis, MO, USA). All other bacterial strains were grown aerobically on brain-heart infusion (BHI) medium (Merck). Agar media were prepared by addition of 1.5% (w/v) agar to the medium; overlay agar contained 0.75% (w/v) agar. Anaerobic conditions were maintained in Oxoid jars containing Anaerocult A (Merck) as a reducing agent.

3.3.2 Antimicrobial screening assay

From each of the selective agar plates (VRBD, NAT, NAV and modified Bile Esculin Agar), 100 colonies were screened for inhibitory substances by the deferred antagonism plate test (Tagg *et al.,* 1976). Briefly, isolates were spot inoculated onto the surface of a non selective agar plate (Table 3) and incubated anaerobically overnight at 37°C. Fifty different isolates arranged in a grid pattern were inoculated onto a single plate. Following incubation, colonies were overlaid with the indicator strains *Escherichia coli* K-12 (49 samples), *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 (47 samples), *Bifidobacterium breve* UCC2003 (21 samples) and *Cronobacter sakazakii* DPC6440 (19 samples). Wilkins Chalgren agar plates were overlaid directly. The overlaid plates were incubated at 37^{o}C for 24 h. Inhibitory activity was demonstrated by a clear zone around the colonies. Colonies surrounded by zones of inhibition were then sub-cultured back onto fresh medium to purify. These colonies were then stocked for later characterisation.

3.3.3 Cell-free-supernatant assay

Antimicrobial activity in the cell-free supernatant (CFS) of the isolates was confirmed using the agar well diffusion method (Ryan *et al.,* 1996). To eliminate inhibition of the

indicator strains being due to acid production, the CFS was adjusted to pH 6.8-7.0 with 1M NaOH then filter sterilised (0.45µM). Antimicrobial activity of the neutralised CFS was assessed against a selection of bacterial strains (*Lb. bulgaricus* LMG 6901, *E. coli* K-12, *B. breve* UCC2003, *C. sakazakii* DPC6440, *Listeria innocua* DPC 3572, *Listeria monocytogenes* DPC3785, *Staphylococcus aureus* DPC 5245, *Salmonella typhimurium* DPC 6452, *S. typhimurium* DPC 6046 and *E. coli* DPC 6239. The antimicrobial activity of *Lb. crispatus* for checked against a further 20 indicator stains (Table 4). Plates were examined for inhibition of growth following 24 h incubation. A clear zone of inhibition was recorded as positive.

3.3.4 Effect of enzymes, heat and pH

The proteinaceous nature of the inhibitor was demonstrated by its sensitivity to the proteolytic enzyme, proteinase K from *Tritirachium album* as described previously (Rea *et al.*, 2010). The neutralised CFS of *Lb. crispatus* was also treated with additional proteolytic enzymes, trypsin, α-chymotrypsin, pronase, protease and pepsin (all enzymes obtained from Sigma).

Filter sterilized catalase (Sigma) was added to the neutralised CFS to evaluate if hydrogen peroxide (H_2O_2) was involved in the antimicrobial activity of the culture supernatants. The CFS was treated with catalase (25mg mL^{-1}) for 3 h at 37°C.

Neutralised CFS (1 mL) was incubated in a heating block at 80°C for 15 and 30 min and at 100°C for 30 min to evaluate the heat stability of the inhibitory substances, The CFS of the *Lactobacillus crispatus* strain was further treated at 45, 50, 55, 60, 65, 70, 75 and 80ºC for 15 min.

The pH of the CFS was adjusted to a selection of values between 2.0 and 12.0 using 1M NaOH and 1M HCl in order to evaluate the effect of pH on the antimicrobial activity of *Lb. crispatus*. These samples were incubated at room temperature for 3-4h.

The residual activity of all treated samples was then assayed against the appropriate indicator strain. Untreated, neutralised CFS was used as a positive control.

3.3.5 Dialysis

In order to estimate the molecular weight of the *Lb. crispatus* antimicrobial, the CFS was subjected to dialysis. Briefly the CFS was placed in cellulose dialysis tubing (12-4,000 Da cut-off; Spectrum® Laboratories) then dialysed against 1 L volumes of phosphate buffered saline at 4°C, with three buffer changes over 24h. The dialysate was tested for the presence of antibacterial activity using the agar well diffusion method, as described above.

3.4 Genetic characterisation of bacterial isolates with anti-microbial properties

3.4.1 Genomic DNA extraction

Cells in 2 mL of an overnight culture were sedimented by centrifugation. DNA was extracted using GenElute^{TM} Bacterial Genomic DNA Kit (Sigma) following the manufacturer's instructions.

3.4.2 Gene sequencing analysis to identify bacterial isolates

The 16S eubacterial ribosomal DNA (rDNA) primers, CO1 and CO2 (Table 5) were used to generate an approximate 1.5-kb 16S rDNA fragment (Simpson *et al.*, 2003). The sequence of the 16S rRNA gene was determined by Sanger sequencing (Beckam Coulter, Essex, UK) and was used to identify the isolates using a National Centre for Biotechnology Information (NCBI) BLAST (basic local alignment search tool) database search (http://blast.ncbi.nlm.nih.gov). Comparison of the 16S rRNA gene sequences obtained using the BLAST program allowed assignment of a strain to a particular species. In general, when 16S rRNA gene similarity values exceed 97%, the strains are considered to belong to the same species (Stackebrandt & Goebel, 1994; Wall *et al.*, 2007). For *Enterococcus* isolates, further PCR analysis was used to distinguish the species present using previously published species-specific primers (Table 5) (Jackson *et al.*, 2004).

3.4.3 Random amplification of polymorphic DNA (RAPD) PCR on *E.coli* isolates

RAPD PCR was performed on the antimicrobial producing *E. coli* isolates in an initial attempt to distinguish and group them. Good discrimination was found with the random primer, P1 (Simpson *et al.*, 2002) which was therefore used in this genetic screen.

3.4.4 Pulsed Field Gel Electrophoresis (PFGE) of bacterial isolates

PFGE analysis was performed using the restriction endonuclease *Xba1*, as previously described (Ribot *et al.,* 2006) to confirm the genetic groupings of *E. coli* isolates exhibiting distinct RAPD PCR fingerprints and to make a final grouping. The *Salmonella* serotype *Braenderup* H9812 standard was used for comparison between gels. For *Lactobacillus* and *Enterococcus* isolates, PFGE was performed as previously described (Simpson *et al.*, 2002) using the *ApaI* enzyme. A low-range molecular weight DNA marker (9.42-194.0 Kb; New England Biolabs, Beverly, MA, USA) was used to determine band size. Electrophoresis was performed in a Bio-Rad CHEF-DR III instrument (Bio-Rad Laboratories, Richmond, CA, USA). For each PFGE pattern, one strain was selected for further analysis.

3.4.5 Bacteriocin-encoding gene sequencing to identify bacteriocins

The presence of bacteriocin encoding genes was studied by PCR amplification with primers specific for the structural genes of previously characterised bacteriocins (Table 5). All PCR reactions were performed using a G-Storm PCR machine (Mason Technologies). PCR products were purified using a QIAquick PCR purification kit (Qiagen) and sequenced by Beckham Coulter Genomics. The DNA sequences were compiled and analysed using the Lasergene 7 software package (DNAstar Inc., Madison, WI). BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to find homologous proteins to the nucleotide coding regions within the nucleotide sequence.

3.5 Matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) Mass Spectrometry to confirm presence of bacteriocins

MALDI-TOF mass spectrometry is a method which allows accurate determination of peptide masses and has been used to confirm the presence of bacteriocins (Mills *et al.,* 2011). Mass spectrometry in all cases was performed with an Axima TOF^2 MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK) as described previously (Field *et al.*, 2008). The antimicrobial peptide of *S. mutans* was detected using colony mass spectrometry. Briefly, colonies were collected from a mMRS agar plate using a sterile plastic loop, mixed with 70% (v/v) propan-2-ol (IPA) containing 0.1% (v/v) trifluoric acid (TFA), vortexed 3 times and centrifuged. The supernatant was removed and used for analysis.

The antimicrobial peptides of *Lb. gasseri* and *Lb. salivarius* were detected using a larger scale preparation - a 40 mL overnight culture of the producing strain. Cells were harvested by centrifugation, resuspended in 70% IPA 0.1% TFA and stirred at room temperature for 3-4 h. Cells were removed by centrifugation and the propan-2-ol was removed by rotary evaporation. The resultant preparation was applied to a Strata C18 solid phase extraction (SPE) column (Phenomenex, Cheshire, UK) pre-equilibrated with methanol and water. The column was washed with 30% (v/v) ethanol and the antimicrobial peptides were subsequently eluted with 70% IPA 0.1% TFA.

The antimicrobial peptide of *E. faecium* was detected in the CFS. A 2L overnight culture of *E. faecium* EM345 N-2 was centrifuged and the supernatant was passed through a column containing 60 g hydrophobic XAD beads (Amberlite XAD16, Phenomenex, Chesire, UK). The column was washed with 30% (v/v) ethanol and the antimicrobial peptides were eluted with 70% IPA 0.1% TFA. The propan-2-ol was removed by rotary evaporation and the resultant preparation was applied to a SPE column as described above. Following elution, the propan-2-ol was removed by rotary evaporation and the sample was applied to a Grace Vydac C8 semi-preparation column (10 mm x 250 mm) running a 25-65% acetonitrile 0.1% (vol/vol) TFA gradient over 45 min. Individual fractions were assayed by well diffusion using the sensitive indicator strain, *L. bulgaricus* LMG 6901. MALDI-TOF mass spectrometry was performed to determine fractions containing the peptides of interest.

3.6 Statistical Methods

The Mann Whitney test was applied using PASW Statistics (version 18), to determine differences in bacterial counts between the elderly group and the non-elderly volunteers. Statistical significance was accepted at $P<0.05$.

4.0 Results

4.1 Isolation and enumeration of anaerobic bacteria

In total, 49 faecal samples (42 ELDERMET samples and 7 non-ELDERMET samples) were obtained for microbiological analysis. Total levels (CFU/g faeces) of cultivable anaerobes, non-spore forming anaerobes (non-clostridial anaerobes), Gram negative anaerobes, *Bacteroides* group and *Enterobacteriaceae* were enumerated on selective

media for 47 of these subjects. Faecal samples from 2 elderly subjects were processed but bacterial counts were not recorded as the samples were not processed within the appropriate time period. There was a large inter-individual variation in the levels of bacteria recovered from faecal samples (Fig. 1). *Enterobacteriaceae* levels showed the most variation with counts ranging from 4.00 to 10.18 $\log_{10}$ CFU/g faeces. Significantly higher numbers of total anaerobes were detected in the non-elderly group than in the elderly group (P<0.05). Median numbers of total anaerobes in the non-elderly group and the elderly group were 10.36 $\log_{10}$ CFU/g faeces and 9.99 $\log_{10}$ CFU/g, respectively.

4.2 Screening anaerobic faecal isolates for anti-microbial production

4.2.1 Screening assay

Bacterial isolates were screened for antimicrobial activity against *Lb. bulgaricus* LMG 6901, *E. coli* K-12, *B. breve* UCC2003 and *C. sakazakii* DPC 6440 (Table 6). *Lb. bulgaricus* LMG 6901 and *E. coli* K-12 were chosen for the initial screening as the former is a Gram positive antimicrobial-sensitive, acid-resistant strain (Casey *et al.,* 2004) while the latter is a Gram negative strain which has been shown to be sensitive to colicins (Gordon and O'Brien, 2006). *B. breve* UCC2003 is a Gram positive gut commensal bacterium while *C. sakazakii* DPC 6440 is a Gram negative pathogen, which has been responsible for illness in infants fed contaminated infant formula (Gurtler *et al.,* 2005).

In total, over 23,000 isolates from 47 faecal samples were screened against *Lb. bulgaricus* LMG 6901. Antimicrobial activity was detected from a total of 49 isolates from 8 different subjects as indicated by zones of clearing in deferred antagonism assays (for example, see Fig. 2), representing an isolation frequency of 0.2% in the initial screening.

Over 24,000 isolates from 49 faecal samples were screened against *E. coli* K-12. Of these, 259 isolates from 12 different subjects produced zones of inhibition against *E. coli* K-12 representing an isolation frequency of 1.1% in the initial screening. For a number of individuals, a high incidence of antimicrobial production among the *Enterobacteriaceae* family was discovered (Table 7). For one subject, EM338, 70 out of

the 74 *Enterobacteriaceae* isolates examined, produced inhibitory activity against *E. coli* K-12. (Fig. 3).

Colonies showing zones of inhibition against *B. breve* UCC2003 and *C. sakazakii* DPC 6440 were not detected in this study.

4.2.2 Cell-free supernatant assay

All the bacterial isolates were collected and purified using general microbiological purification methods. Antimicrobial activity was confirmed in the neutralised CFS of 62 isolates (42 with activity against *E. coli* K-12 and 20 with activity against *Lb. bulgaricus* LMG 6901) which demonstrated the presence of potential bacteriocins.

4.3 Characterisation of bacterial isolates with anti-microbial properties

4.3.1 Identification of bacterial isolates

The 62 antimicrobial producers were identified through analysis of the 16S rRNA DNA sequence of each isolate. *Enterococcus* strains had their identity confirmed using species-specific PCR amplification. Isolates were identified as follows: *Lb. salivarius* (10), *Lb. gasseri* (4), *Lb. crispatus* (2), *Enterococcus faecium* (3), *Streptococcus mutans* (1), and *E. coli* (42).

4.3.2 RAPD PCR on *E. coli* isolates

RAPD analysis was performed on the antimicrobial-producing *E. coli* isolates. Primer P1 generally generated 5–7 DNA fragments ranging in size from 100 bp to 1.5 kb per isolate. RAPD PCR was performed on the 42 *E. coli* isolates obtained from the 12 elderly subjects. RAPD PCR grouped these isolates into approximately 15 genotypes (Fig. 4) and the profiling revealed that most of the faecal samples potentially contained one dominant strain type. Only four of the subjects appeared to harbour multiple strains, with sample EM338 having what appeared to be 3 genetically distinct strains and samples, EM357, EM362 and EM371 each having two genetically distinct strains. In addition, isolates from two separate individuals with the same RAPD PCR profile were found on only two occasions.

4.3.3 PFGE strain discrimination of *E. coli* isolates

PFGE has been shown to have a higher discriminatory power than RAPD-PCR. Therefore, to confirm the genetic groupings of all faecal isolates and to make a final grouping, PFGE analysis was performed using the restriction endonuclease, XbaI. PFGE grouped the isolates into approximately 11 genotypes (for example, see Fig. 5). Indeed a number of isolates which appeared to have different RAPD fingerprinting patterns had identical PFGE fingerprinting patterns. In another case, two strains which appeared to have the same RAPD fingerprinting pattern showed different patterns when PFGE was performed.

This analysis revealed that most of the faecal samples potentially contained one dominant strain type. One subject harboured two different strains. Isolates from two separate individuals with the same PFGE profile were found on only two occasions.

4.3.4 PFGE strain discrimination of *Lactobacillus/Enterococcus* isolates

PFGE analysis (using Apa1 restriction endonuclease) was performed on the antimicrobial-producing *Lactobacillus* (16 strains) *and Enterococcus* strains (3 strains) to distinguish and group them. PFGE analysis grouped *Lactobacillus* strains into 3 genotypes (for example, see Fig. 6). This analysis revealed that subject EM307 contained one dominant antimicrobial producing *Lb. salivarius* strain type. In addition, this particular strain was also found in subject EM366. As regards the antimicrobial-producing *Lb. gasseri*, one strain was found in both subject EM350 and subject EM351. Similarly, a single *E. faecium* was found in two subjects (EM345 and SP006)

4.3.5 Effect of enzymes, heat and pH

Treatment of the CFS with the proteolytic enzyme, proteinase K resulted in loss of antibacterial activity suggesting the antimicrobials have a proteinaceous moiety (Table 8). However, for the *Lb. crispatus* strains there was no loss of antimicrobial activity. The CFS of this strain was further treated with additional proteolytic enzymes, pepsin, pronase and protease and these also failed to eliminate activity. α-chymotrypsin and trypsin both inhibited the indicator strain and were therefore deemed inappropriate for use. The antimicrobials of a number of bacterial strains (*E. coli* and *Lb. crispatus*) were found to be heat labile, others were found to be heat stable up to 100ºC for 30 min

(Table 8). Further investigation revealed that the antimicrobial activity of *Lb. crispatus* was lost after heat treatment at ~60ºC for 15 min. Furthermore, adjustment of the pH of the CFS of *Lb. crispatus* resulted in loss of antimicrobial activity at pH 2.0 and pH 11.0. The antimicrobial activity remained in membrane dialysis tubing with a cut-off pore size of 12-14 kDa indicating it was large in size.

4.3.6 Inhibitory Spectrum

The inhibitory spectrum of the 16 genetically distinct strains (*Lb. gasseri, Lb. salivarius, Lb. crispatus, E. faecium, S. mutans* and *E. coli* strains) was investigated. The *Lb. salivarius* and the *E. faecium* isolates were shown to inhibit *L. innocua* and *L. monocytogenes* in addition to *Lb. bulgaricus* (Table 9). *Lb. crispatus* did not inhibit any of the additional indicator strains used.

4.3.7 Identification of bacteriocins

The presence of structural genes for a number of known bacteriocins was identified in the DNA of the isolates and the actual production of the bacteriocin was confirmed using MALDI-TOF mass spectrometry. For example, PCR and amplicons sequence analysis of *Lb. gasseri* confirmed the presence of the structural gene for gassericin T. MALDI-TOF mass spectrometry was then used to confirm the presence of a peptide of 5542 Da which corresponds to the molecular mass gassericin T (http://bactibase.pfba-lab-tun.org/BAC117) (see Fig. 7). Table 9 displays a summary of the faecal isolates exhibiting antimicrobial activity.

5.0 Discussion

In the present study, we investigated the culturable microbiota of 42 elderly subjects (≥65 yrs) participating in the ELDERMET project and 7 non-elderly volunteers (22-57 yrs). Culture-based techniques have been routinely used to determine the bacterial composition of faeces (Hopkins *et al.,* 2001; O'Sullivan *et al.,* 2011) and the estimate of cultivability of gastrointestinal bacteria is approximately 10–50% (Adlerberth and Wold, 2009; O'Toole and Claesson, 2010)**.** It has recently been reported that the majority of intestinal bacterial taxa are actually culturable under controlled anaerobic conditions and that usage of culture media that more accurately replicates the gut environment will facilitate further exploration of the human gut microbiota *in vitro* (Goodman *et al.,* 2011). The bacterial numbers recovered in this study correspond well with previous culture-based studies on human faecal microbiota (Bouhnik *et al.,* 2007; Delgado *et al.,* 2006; Guigoz *et al.,* 2002; Hayashi *et al.,* 2002; Holdeman *et al.,* 1976; Hopkins *et al.,* 2001; Mättö *et al.,* 2005; Meijer-Severs & Van Santen, 1986; O'Sullivan *et al.,* 2011; Woodmansey *et al.,* 2004). The non-elderly volunteers had significantly more total anaerobes in their faeces compared to elderly subjects. Indeed, it has been widely documented that the elderly microbiota differs from that of younger adults (Claesson *et al.,* 2011) and just under half of our elderly group were on antibiotics which have been shown to dramatically disturb the composition of the microbiota (Woodmansey *et al.,* 2004). However, there was no significant difference in the levels of cultivable non-spore forming anaerobes, Gram negative anaerobes, *Enterobacteriaceae* and *Bacteroides* between the elderly and the non-elderly group.

Faecal bacteria were screened for isolates with the ability to inhibit the growth of 1) a *Lactobacillus bulgaricus* strain, 2) a *Bifidobacterium breve* strain, 3) an *E. coli* strain and 4) a *Cronobacter sakazakii* strain. Although this study was extensive, a limited number of bacteriocin-producing bacteria were isolated. We recovered 49 isolates (from 8 subjects) with inhibitory activity against *Lb. bulgaricus* LMG 6901 (0.2% frequency) and 20 of these isolates had activity in the cell-free supernatant (CFS). Two hundred and fifty nine isolates (from 12 subjects) displayed inhibitory activity against an *E. coli* K-12 (1.1% frequency) and 42 of these isolates showed activity in the CFS. No isolates were shown to inhibit *B. breve* UCC2003 and *C. sakazakii* DPC 6440. Indeed, only a limited number of bacteriocin producing-*Bifidobacterium* strains have been isolated

previously (Martinez *et al.,* 2013). Additionally, *B. breve* UCC2003 has been shown to produce EPS (Fanning *et al.,* 2012) which may offer some protection to cells against antimicrobial agents (Sutherland, 2001). *Cronobacter* on the other hand, is not typically found in the normal mammalian intestinal tract, which may be a plausible reason as to why no bacteriocins of intestinal origin were found to target this pathogen in the present study.

The frequency of isolation of antimicrobial producing isolates between elderly and non-elderly subjects and antibiotic-treated and non-antibiotic treated individuals were compared. Isolates derived from elderly faecal samples showed more inhibitory activity against *E. coli* compared to the isolates derived from non-elderly subjects. Antimicrobial producing isolates with activity against *Lb. bulgaricus* were more frequently isolated from non-antibiotic treated individuals compared to antibiotic-treated individuals. Nonetheless, these observations should be interpreted with caution given the limited number of non-elderly subjects that were sampled in this study.

The overall low frequency of isolation of bacteriocin-producing bacteria found in the present study may be due to number of inherent limitations incurred by using culture-based screening methods (O'Shea *et al.,* 2009): 1) the genes responsible for bacteriocin production are typically tightly regulated – therefore, bacteriocin production may go undetected under conditions where the responsible operons are switched off (Kleerebezem, 2004); 2) bacteriocins generally have a narrow spectrum of inhibitory activity and might not be detected using only a low number of indicator strains for screening (De Vuyst *et al.,* 2004) and; 3) culture-based methods can limit the screening study to the "easy-to-culture" intestinal bacteria. Therefore these culture methods are likely to only allow for the detection of a small fraction of potential bacteriocin-producing bacteria in the intestine. In order to overcome these limitations, culture-independent, genomic mining tools, such as BAGEL, may be employed more frequently in the future (de Jong *et al.,* 2010; De Jong *et al.,* 2006).

In the present study, 42 *E. coli* isolates were found to inhibit *E. coli* K-12 in the CFS. Although *E. coli* does not represent a major group of bacteria of the intestinal microbiota, it is a normal and ecologically important inhabitant of the human gut (Blum-Oehler *et al.,* 2003). Molecular fingerprinting using PFGE revealed 11 genetically distinct *E. coli* strains, indicating a high incidence of repeated isolation of

antimicrobial producing strains, particularly within individual intestinal samples. Indeed for one subject, 70 out the 74 *Enterobacteriaceae* isolates inhibited the indicator stain *E. coli* K12. This high incidence of an antimicrobial producing *E. coli* in the gut supports the theory of dominance within complex ecosystems as a consequence of antimicrobial production.

Although the antimicrobial compounds produced by the *E. coli* strains were not identified, it is likely that these heat-labile peptides are colicins or microcins, well characterised bacteriocins produced by and active against strains of *E. coli* and several related species in the *Enterobacteriaceae* family (O'Shea *et al.,* 2012). Colicins have a narrow spectrum mainly targeting strains of the same species, whereas microcins have activity against a wider spectrum of strains (Šmajs *et al.,* 2012). To date, approximately 26 colicins and 14 microcin types have been identified (Rebuffat, 2011; Šmajs *et al.,* 2010). In a recent paper, Smajs *et al*. (2010) claimed that 55% of over 411 *E coli* isolates from human sources produced colicins. Gordon & O'Brien (2006) found that 38% of human *E. coli* strains produce colicins. Although the ecological meaning of colicinogeny is largely unknown (Gordon & O'Brien, 2006), it is generally believed that colicins participate in the intraspecific or interspecific competition in the colon microbiota facilitating its introduction into novel habitats (Šmarda & Obdržálek, 2001).

The 42 isolates with activity against *Lb. bulgaricus* LMG 6901 were reduced to 5 genetically distinct strains through further characterisation. One of the isolates, *E. faecium,* was found to have inhibitory activity against *Lb. bulgaricus* LMG 6901, *L. innocua* DPC 3572 *and L. monocytogenes* DPC 3785. Enterococci are natural inhabitants of the GIT and produce an impressive array of different bacteriocins (Franz *et al.,* 2007). In the present study, the *E. faecium* was found to produce both enterocin L-50 and enterocin P, as described previously (Cintas *et al.,* 2000).

One isolate, *Streptococcus mutans* which inhibited growth of *Lb. bulgaricus* LMG 6901 was found to product mutacin II. *S. mutans* is generally not associated with gut derived organisms but is found to inhabit the oral cavity where it is generally accepted as one of the principal aetiological agents of dental caries (Kamiya *et al.,* 2005). A previous study by our group also isolated a mutacin II-producing *S. mutans* strain from another elderly faecal sample (Lakshminarayanan *et al.,* 2013). Mutacins are likely to confer an ecological advantage in diverse bacterial communities (Kamiya *et al.,* 2005). Indeed,

researchers have found a strong correlation between the ability of a *S. mutans* strain to colonise the oral cavity and the production of the bacteriocin mutacin 1140 (Hillman, 2002).

A number isolates with inhibitory activity against *Lb. bulgaricus* LMG 6901 were identified as lactobacilli. *Lactobacillus* spp. are thought to play a fundamental role in stabilization of the microflora by providing an important microbial defence against intestinal colonisation by exogenous pathogenic microorganisms (Song *et al.,* 1999). This screening study resulted in the isolation of a *Lb. gasseri* strain producing the bacteriocin, gassericin T. *Lb. gasseri* appears to be one of the main species of lactobacilli that inhabits the human GIT (Delgado *et al.,* 2007) and gassericin T is a broad-spectrum bacteriocin which has previously been isolated from human faeces (Kawai *et al.,* 2000; Lakshminarayanan *et al.,* 2013). Another faecal isolate, *Lb. salivarius,* showed inhibitory activity against *Lb. bulgaricus* LMG 6901, *L. innocua* DPC 3572 *and L. monocytogenes* DPC 3785 and was found to produce ABP-118. ABP-118, a two component class II bacteriocin produced by the probiotic bacterium *Lb. salivarius* subsp. *salivarius* UCC118, was originally isolated from a human intestinal tract. Most notably, Corr *et al.* (2007) found that *Lb. salivarius* UCC118 provided protection against *L. monocytogenes* in mice and this inhibition was shown to be the direct results of the production of ABP118.

A *Lb. crispatus* strain with inhibitory activity against *Lb. bulgaricus* LMG 6901 was also isolated in this study. *Lb. crispatus* is one of the predominant hydrogen peroxide (H_2O_2) producing species found in the vagina and is under development as a probiotic for the treatment of bacterial vaginosis (Antonio & Hillier, 2003; Hemmerling *et al.,* 2010). *Lb. crispatus* has also been previously isolated from faecal samples (Song *et al.,* 1999) and the protective effects of dietary supplementation of *Lb. crispatus* on experimentally induced colitis in mice has been investigated (Castagliuolo *et al.,* 2005). The antimicrobial activity appeared to be heat labile, narrow spectrum and insensitive to protease inactivation in the CFS. In addition, the antimicrobial agent appeared to be large (>10 kDa), which is atypical of Class I and Class II bacteriocins (Cotter *et al.,* 2005). A bacteriocin produced by a *Lb. crispatus,* crispacin A, has been characterised (Tahara and Kanatani, 1997). However, in contrast to the antimicrobial isolated in the present study, crispacin A was small in size (5393 Da), heat stable and sensitive to

proteolytic enzymes. Further work will aim to identify and characterise the antimicrobial compound produced by *Lb. crispatus.*

In conclusion, this screening system resulted in the isolation of a number common intestinal species (*E. coli, Lb. salivarius, E. faecium*) with antimicrobial activity, but more significantly also selected for *S. mutans*, a species not normally detected among the human intestinal microbial community. Compounds that inhibit growth or kill gastrointestinal pathogens such as *L. monocytogenes* were produced by bacteria residing in the intestine of elderly Irish subjects. Interestingly, a *Lactobacillus crispatus* strain (EM-LC1) was isolated which was found to produce a potentially novel heat labile antimicrobial compound which was resistant to protease activity in the cell-free supernatant. The present work increases our knowledge regarding antimicrobial production among intestinal bacteria and suggests that perhaps the culture-based isolation of antimicrobials is now limited in its potential to isolate novel bacteriocin-producers as indicated by the repeated isolation of previously characterised bacteriocins in this extensive screening study.

Table 1. Stratification of ELDERMET subjects (n=42)

Major property	**Details**	**No. of subjects**
Community	subjects living independently in the community.	**7**
Day hospital/Out-patients	subjects from the Elderly Services Day Hospital, St. Finbarr's Hospital & Out-Patients Clinic, Cork University Hospital.	**12**
Antibiotic-treated Community	subjects living independently in the community who have received an oral antibiotic within 4 weeks of collection of sample.	**17**
Rehab/In-patients	community-dwelling subjects, recruited from the rehabilitation wards during their in-patient stay.	**4**
Clostridium difficile-infected	subjects hospitalised with *Clostridium difficile*–associated diarrhoea (CDAD)	**2**

Table 2. Growth medium used to enumerate anaerobic bacteria from faecal samples

Agar	**Selective for**	**Incubation Time**
Wilkins-Chalgen (WC) anaerobe agar[a]	Total anaerobes	48h
NAT medium (Wilkins Chalgren with N-S anaerobe selective supplement)[b] + 0.1% Tween 80 + 5% (v/v) defibrinated horse blood[c]	Non-spore forming anaerobes	48h
NAV medium (Wilkins Chalgren with G-N anaerobe selective supplement)[d] + 5% (v/v) defibrinated horse blood[c]	Gram negative anaerobes	48h
Violet Red Bile Dextrose (VRBD) agar[e]	Total *Enterobacteriaceae*	24h
Bile Esculin Agar (BEA)[f] with 100 µg/mL gentamicin[f], 7.5 µg/mL vancomycin[g] and 10µg/mL Hemin[f]	*Bacteroides*	72h

[a]Oxoid Ltd; [b]Oxoid Ltd. The supplement contains nalidixic acid as the selective agent. The medium is particularly useful for the recovery of non-sporing Gram-positive anaerobes since the presence of 'Tween 80' stimulates their growth; [c]Cruinn Diagnostics, Dublin, Ireland; [d]Oxoid Ltd. The supplement contains nalidixic acid and vancomycin as the selective agents making the medium selective for Gram negative bacteria; [e]Merck; [f]Sigma. Selective inhibition of facultative anaerobes and most gram-negative anaerobic organisms is obtained by the presence of gentamicin and bile; [g]Duchefa Biochemie, Haarlem, The Netherlands

Table 3. Media used for the deferred antagonism plate test

Isolation Media:	**Re-spot 100 colonies on to:**
VRBD agar	Brucella Agar[a] supplemented with Hemin[b] (5µg/mL) and Vitamin K1[b] (1µg/mL)
NAT medium	Wilkins-Chalgren (WC) anaerobe agar[b]
NAV medium	Wilkins-Chalgren (WC) anaerobe agar
Modified Bile Esculin Agar (BEA)	Brucella Agar supplemented with Hemin (5µg/mL) and Vitamin K1 (1µg/mL)

[a]Beckton Dickinson Microbiology Systems. Cockeysville, Md. [b]Sigma

Table 4. Indicator strains used for characterisation of *Lb. crispatus* antimicrobial

Indicator Strain	**Culture Medium**	**Growth Temperature (ºC)**	**Condition**
Lactococcus lactis HP	GM17	30	Aerobic
Lactococcus lactis MG1363	GM17	30	Aerobic
Enterococcus casseliflavus 5053	GM17	37	Aerobic
Enterococcus durans 5133	GM17	37	Aerobic
Enterococcus faecium 5119	GM17	37	Aerobic
Staphylococcus aureus DPC5247	BHI	37	Aerobic
Streptococcus agalactiae ATCC 13813	BHI	37	Aerobic
Lactobacillus salivarius AH4231	MRS	37	Anaerobic
Lactobacillus salivarius UCC119	mMRS	37	Anaerobic
Lactobacillus salivarius UCC118	mMRS	37	Anaerobic
Lactobacillus fermentum L34	mMRS	37	Anaerobic
Lactobacillus plantarum WCFS1	mMRS	37	Anaerobic
Lactobacillus plantarum NCIMB 8826	mMRS	37	Anaerobic
Lactobacillus paracasei B636-43364	mMRS	37	Anaerobic
Lactobacillus casei Shirota	mMRS	37	Anaerobic
Lactobacillus sakei LMG2313	mMRS	37	Anaerobic
Lactobacillus reuteri DSM20016	mMRS	37	Anaerobic
Lactobacillus acidophilus ATCC4356	mMRS	37	Anaerobic
Lactobacillus johnsonii DSM 20553	mMRS	37	Anaerobic
Lactobacillus rhamnosus CCUG 36679	mMRS	37	Anaerobic

Table 5. Primers used in this study

Primer*	Sequence (5′to 3)	Specificity	Amplicon size (bp)	Annealing temp. (ºC)	References
CO1	AGTTTGATCCTGGCTCAG	16S rRNA gene	1500	50	(Simpson *et al.*, 2003)
CO2	TACCTTGTTACGACT				
P1	ACGCGCCCT	RAPD primer	-	36	(Simpson *et al.*, 2002)
Gass T – F	TGGATTTAAATTGCCTGAAAC	Gassericin T gene	645	58	(Lakshminarayanan *et al.*, 2013)
Gass T – R	CATTCCCCCACTTGTTTCC				
118α - F	ATGATGAAGGAATTTACAG	ABP 118 gene	700	50	(Barrett *et al.*, 2007)
118im -R	CCACGCTCTCACATAAC				
FM1	GAAAAAACAATAGAAGAATTAT	*E. faecium* sodA gene	215	55	(Jackson *et al.*, 2004)
FM2	TGCTTTTTTGAATTCTTCTTTA				
Mutacin II – F	AACGCAGTAGTTTCTTTGAA	Mutacin II gene	444	52	(Kamiya *et al.*, 2005)
Mutacin II – R	TTCCGGTAAGTACATAGTGC				
Ent P – F	GATGCAGCTACGCGTTCATATGG	Enterocin P gene	138	56	This study
Ent P – R	ATGTCCCATACCTGCCAAACCA				
Ent L-50 – F	GTGGAAAGCTAGTATTTGCAAC	Enterocin L-50 gene	511	49	(Dezwaan *et al.*, 2007)
Ent L-50 – R	AGCGTTAAGCCGAATGTTTACAC				

*Primers were obtained from Sigma

Table 6. Frequency of isolation of antimicrobial producing isolates from elderly and non-elderly individuals against Gram positive and Gram negative indicator strains

		***E. coli* K-12**	***Lb. bulgaricus* LMG 6901**	***B. breve* UCC 2003**	***C. sakazakii* DPC 6440**
Total subjects (*n* 49)	**No. of Isolates**	**259/24420 (1.06%)**	**49/23180 (0.21%)**	**0/9361**	**0/9230**
	No. of subjects	**12/49 (24.50%)**	**8/47 (17.02%)**	**0/21**	**0/19**
Elderly subjects (*n* 42)	No. of Isolates	259/20720 (1.25%)	48/20580 (0.23%)	0/9061	0/9230
	No. of subjects	12/42 (28.57%)	7/42 (16.67%)	0/20	0/19
Antibiotic group (*n* 18)	No. of Isolates	30/8862 (0.34%)	2/9044 (0.02%)	-	-
	No. of subjects	5/18 (27.78%)	1/18 (5.56%)	-	-
Non-antibiotic group (*n* 24)	No. of Isolates	229/11858 (1.93%)	46/11536 (0.4%)	-	-
	No. of subjects	7/24 (29.17%)	6/24 (25%)	-	-
Non-elderly subjects (*n* 7)	No. of Isolates	0/3700	1/2600 (0.04%)	0/300	0/0
	No. of subjects	0/7	1/5 (20.0%)	0/1	0/0

Table 7. Subjects (*n* 12) showing inhibitory activity against *E. coli* K-12 within the *Enterobactericeae* family

Subject	**Category**	***Enterobactericeae* Count[a]**	**Colonies picked for screening**	**No. of colonies producing zones of inhibition[b]**	**Frequency %**
EM338	Rehab	6.8×10^6	74	70	94.6
EM350	Rehab	8.9×10^6	100	6	6
EM352	Community (antibiotic)	1.3×10^8	100	1	1
EM354	Day Hospital	2.9×10^7	100	3	3
EM355	Day Hospital	5.2×10^5	50	25	50
EM357	Day Hospital	1.7×10^7	100	41	41
EM362 (T3)	Community	1.4×10^7	100	63	63
EM371	Day Hospital	9.7×10^8	100	6	6
EM372	Community (antibiotic)	7.8×10^7	75	6	8
EM376	Community (antibiotic)	5.6×10^6	50	9	18
EM377	Community (antibiotic)	2.9×10^7	100	7	7
EM381	Community (antibiotic)	5.1×10^7	50	3	6

[a]CFU/g faeces; [b]zones of inhibition against the indicator strain *E. coli* K-12

Table 8. Effect of enzymes and heat treatment on antimicrobial activity in anaerobic isolates

Strain ID	Identification	No treatment	Proteinase K treatment	Heat treatment		
				80ºC x 15 min	80ºC x 30 min	100ºC x 30 min
EM338 V-8	*E. coli*	9	-	-	-	-
EM350 V-2	*E. coli*	10	-	-	-	-
EM352 V-1	*E. coli*	13	-	-	-	-
EM354 V-1	*E. coli*	13	-	8	8	-
EM355 V-1	*E. coli*	11	-	7	6.5	-
EM357 V-1	*E. coli*	13	-	-	-	-
EM362 V-2	*E. coli*	9	-	6.5	S	-
EM371 V-1	*E. coli*	9	-	-	-	-
EM372 V-1	*E. coli*	10	-	-	-	-
EM376 V-1	*E. coli*	10	-	S	-	-
EM381 V-1	*E. coli*	10	-	-	-	-
EM345 N-1	*E. faecium*	13	-	12	12	12
EM350 N-1	*Lb. gasseri*	16	-	14	14	14
EM307 NAT-1	*Lb. salivarius*	15	-	14	14.5	14.5
EM307 WC-1	*S. mutans*	10	-	10	9	11.5
EM367 WC-2	*Lb. crispatus*	10	10	-	-	-

The diameters of the zones of inhibition, including the diameter of the well, which was approximately 5 mm; -: no zone of inhibition; S: slight zone of inhibition around periphery of well

Table 9. Overview of faecal isolates exhibiting antimicrobial activity

Strains [a]	Strain IDs	Activity against	Heat stability	Enzyme sensitivity	Bacteriocin Identification[b]
Lb. salivarius	EM307NAT1	*Lb. bulgaricus* LMG 6901, *L. innocua* DPC 3572, *L. monocytogenes* DPC 3785	stable	sensitive to proteinase K	ABP-118
Lb. crispatus	EM-LC1	*Lb. bulgaricus* LMG 6901	labile	resistant to proteinase K, protease and pepsin	ND
Lb. gasseri	EM350N1	*Lb. bulgaricus* LMG 6901	stable	sensitive to proteinase K	Gassericin T
E. faecium	EM345N1	*Lb. bulgaricus* LMG 6901, *L. innocua* DPC 3572, *L. monocytogenes* DPC 3785	stable	sensitive to proteinase K	Enterocin L-50 A/B & Enterocin P
S. mutans	EM307WC1	*Lb. bulgaricus* LMG 6901	stable	sensitive to proteinase K	Mutacin II
E. coli	EM338V8, EM350V2, EM352V1, EM354V1, EM355V1, EM357V1, EM362V2, EM371V1, EM372V1, EM376V1, EM381V1	*E. coli* K-12	labile	sensitive to proteinase K	ND

[a] identified by 16S rRNA gene analysis; [b] identified by bacteriocin structural gene sequencing and confirmed using MALDI-TOF-MS; ND: not determined

Figure 1. Levels of total anaerobes (yellow boxes), non-spore forming anaerobes (blue boxes), Gram negative anaerobes (red boxes), *Bacteroides* (purple boxes) and total *Enterobacteriaceae* (green boxes), in faecal samples from 40 elderly subjects and 7 non-elderly subjects +, mean value; *, P<0.05 comparisons between elderly and non-elderly subjects (Mann-Whitney test)

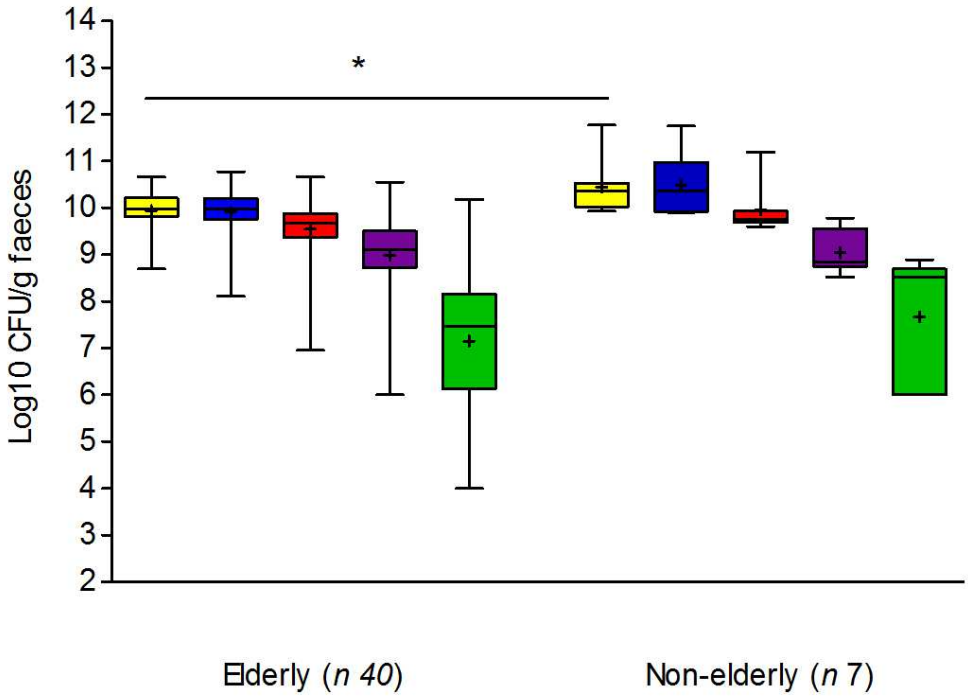


Figure 2. Faecal isolates (from NAT plate) showing zones of inhibition against *L. bulgaricus* LMG 6901

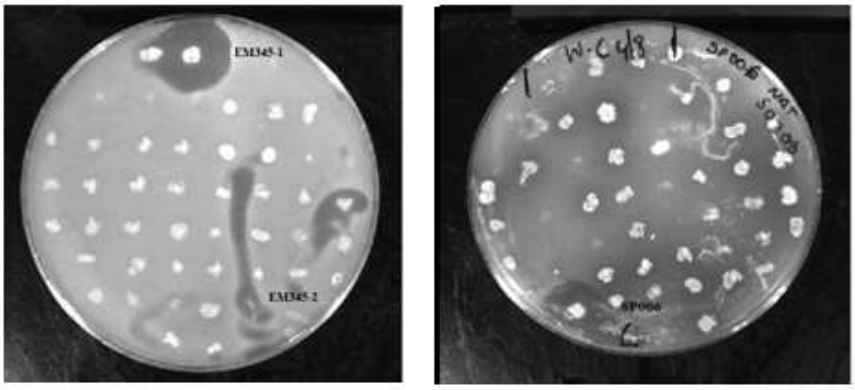


Figure 3. VRBD isolates from one subject (EM338) showing zones of inhibition against *E. coli* K-12

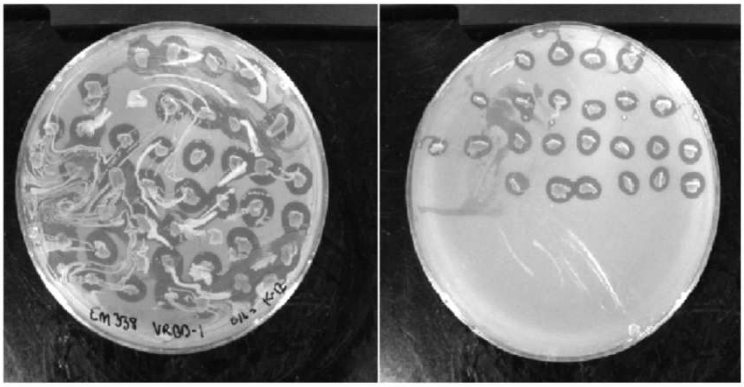


Figure 4. Genetic fingerprinting by RAPD PCR of *E. coli* isolates. This gel illustrates 14 of the 15 RAPD profiles from 9 different subjects. (M = molecular weight marker)

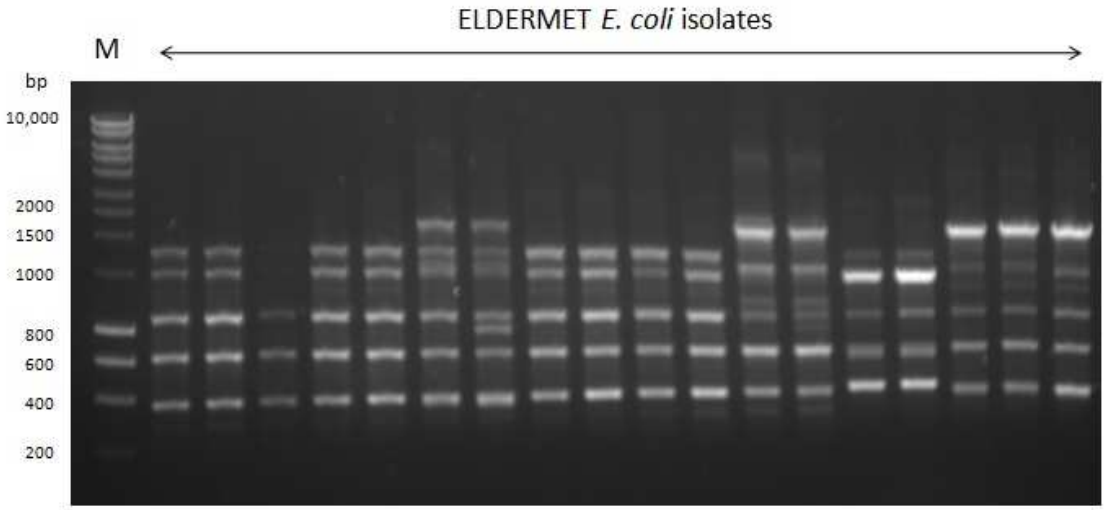


Figure 5. PFGE profiles generated using *XbaI* of a selection of ELDERMET *E. coli* isolates, with antimicrobial activity against *E. coli* K-12. (M = molecular weight marker, *Salmonella* serotype *Braenderup* H9812)

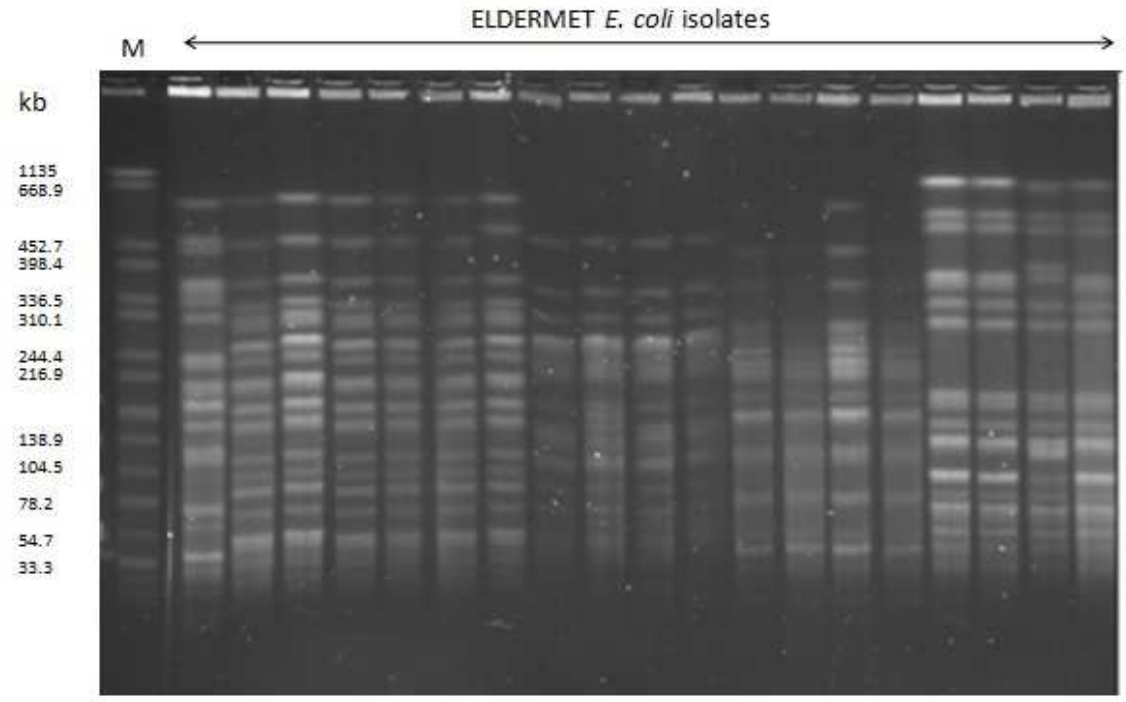


Figure 6. PFGE profiles of a selection of ELDERMET *Lactobacillus* isolates, with anti-microbial activity against *Lb. bulgaricus* LMG6901 (M = molecular weight marker; *Apa1* restriction enzyme)

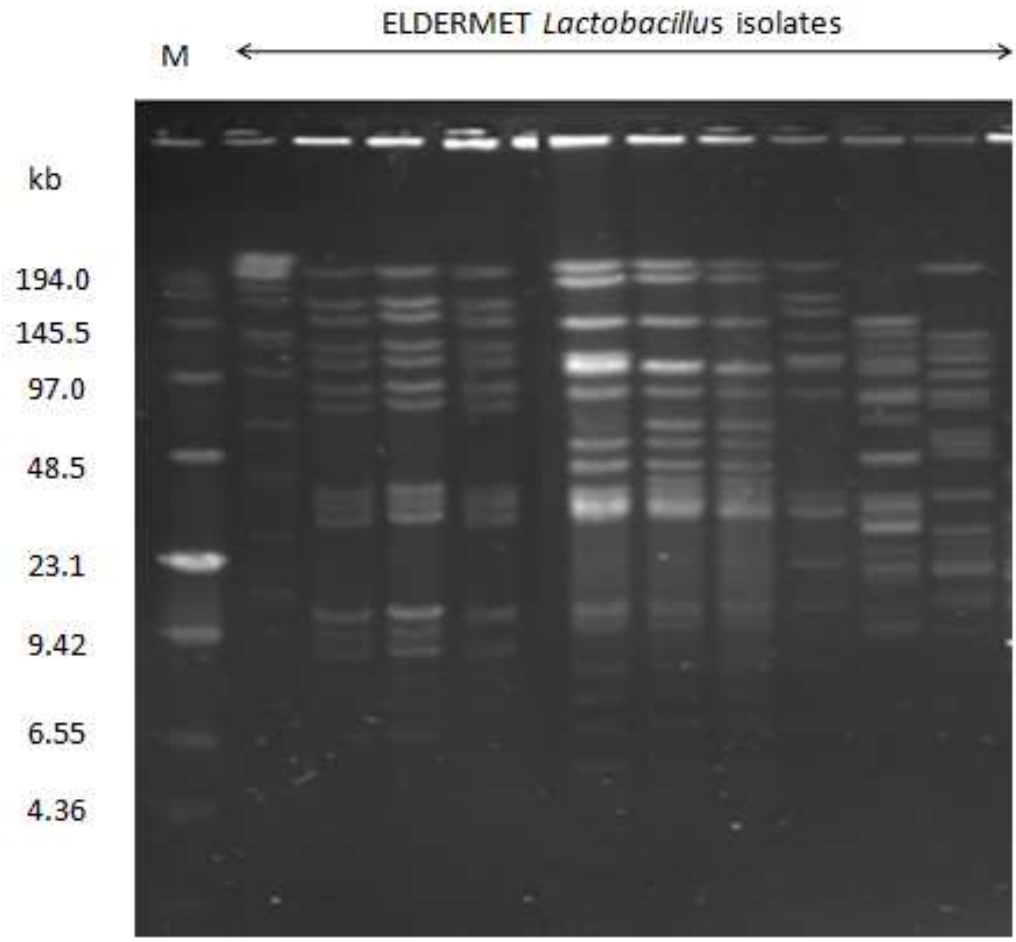
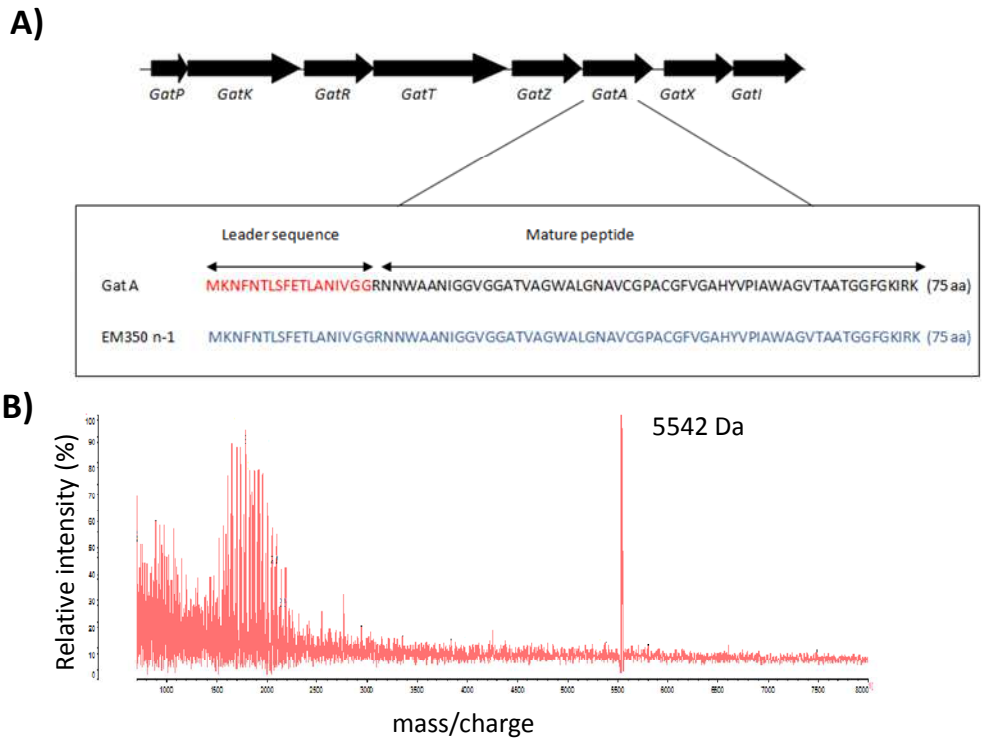


Figure 7. A) Genetic organization of a segment of the gassericin T biosynthetic gene cluster, including the structural genes (Gat A) and the predicted peptide sequence of the inhibitor from *Lb. gasseri* EM350 N-1. B) MALDI-TOF-mass spectrometry data of gassericin T produced by *Lb. gasseri* (EM350 N-1)



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CHAPTER 7

Draft genome sequence of *Lactobacillus crispatus* EM-LC1, an isolate with antimicrobial activity cultured from an elderly subject

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1.0 Abstract

Lactobacilli are Gram positive prokaryotes that naturally colonise the human gastro-intestinal tract (GIT) and vagina. Although not numerically dominant in the complex intestinal microbiota, they are considered as key commensals that promote a healthy GIT. The draft genome sequence of an elderly-derived faecal isolate, *Lb. crispatus* EM-LC1, which exhibited antimicrobial activity *in vitro*, was determined. The genome is composed of 1,862,161 bp with a G+C content of 36.9 %. It is predicted to contain 1,827 putative coding DNA sequences (CDSs) representing an 87.6% coding density. The genome contains 45 transfer RNA genes. No rRNA locus was assembled due to exclusion of repetitive sequence from the assembly. *In silico* analyses predicted 26 transposase genes and a locus for a type I R/M system. Considerable transporter and fermentative capacities expected for organisms residing within the nutrient-rich conditions of the GIT were encoded on the genome. Several mucus-binding proteins, S-layer proteins and a fibronectin-binding protein, implicated in adhesion to human intestinal cells, were identified. Two scaffolds were identified which bear the characteristics of integrated plasmids. Analysis of these two regions revealed the presence of a toxin/antitoxin system, of the zeta/epsilon family. Among the *Lb. crispatus* species with publically available genome sequences, *Lb. crispatus* ST1 is the only strain with a complete genome sequence available. The comparison of EM-LC1 to ST1 revealed 135 protein-encoding genes that were present in EM-LC1 but not in ST1. Among them are genes related to bacteriocin (helveticin) production, genes that code for a toxin/antitoxin system as well as a large number of genes encoding hypothetical proteins.

2.0 Introduction

The lactic acid bacteria (LAB) are low G+C, Gram positive bacteria that produce lactic acid as the major fermentation end-product of sugars (Lahtinen *et al.,* 2011). The LAB include a number of genera, the largest of which is the genus *Lactobacillus*. The lactobacilli encompass a considerable number of different species that display a relatively large degree of diversity (Claesson *et al.,* 2007). A recent taxonomic revision of the *Lactobacillus* genus, based on 16S rRNA sequence analysis, indentified over 150 species (Salvetti *et al.,* 2012). Members of this genus are described as fastidious organisms frequently found in nutritionally-rich environments such as food and soil as well as the mouth, gastrointestinal and genital tract of humans and many animals (Salvetti *et al.,* 2012). Indeed, the lactobacilli are considered a subdominant member of the human gut microbiota and some species are considered to have probiotic properties, offering a beneficial role in maintaining the health status of the host (Klaenhammer, 2000). The most well known members of this group are classified as the 'acidophilus complex', composed of several species of closely related lactobacilli that have historically been isolated from the gastrointestinal tract of humans and animals (Altermann *et al.,* 2005). They include *Lb. acidophilus, Lb. gasseri, Lb. johnsonii, Lb. amylovorus, Lb. gallinarum* and *Lb. crispatus* (Altermann *et al.,* 2005; Forde *et al.,* 2011). Of these, *Lb. acidophilus* remains to be one of the most widely recognised and is a commercially distributed probiotic culture (Altermann *et al.,* 2005). However more recently, interest in *Lb. crispatus* has been increasing. Indeed, *Lb. crispatus* can persist in the gastrointestinal tract (Walter, 2008) and is among the most prevalent species of the *Lactobacillus*-dominated human vaginal microbiota (Ma *et al.,* 2012; Witkin *et al.,* 2007). *Lb. crispatus* strain CTV-05, a vaginally derived H_2O_2-producing strain, is a probiotic that is currently being evaluated for the treatment and prevention of bacterial vaginosis (Hemmerling *et al.,* 2010).

LAB are renowned for their production of antimicrobial peptides (bacteriocins), which display great diversity with respect to structure and mode of action varying from extensively post-translationally modified lantibiotics such as nisin to large unmodified heat labile proteins such as helveticin (De Vuyst, 2012). Bacteriocin production may be particularly important as killing-peptides *in vivo*. *Lactobacillus salivarius* UCC118 produces a bacteriocin *in vivo* that was found to provide significant protection to mice

against infection with *Listeria monocytogenes* (Corr *et al.,* 2007). Bacteriocin production may also confer a competitive advantage to the producing strain by allowing it to dominate complex microbial populations (O'Shea *et al.,* 2009). Hence, bacteriocin production is considered a probiotic trait as it may aid probiotic survival within the intestine (Dobson *et al.,* 2012).

The determination of the complete genome sequence of an organism represents a crucial step in moving towards a better understanding of the organism in question. *Haemophilus influenza* was the first available microbial genome sequence (Fleischmann *et al.,* 1995). Since then, the number of complete genome sequences is increasing rapidly, due to the availability of next-generation sequencing technologies at affordable costs. The genomes of over 200 *Lactobacillus* strains have been sequenced according to the Genomes Online Database (GOLD; www.genomesonline.org). These genome sequencing projects have paved the way for an improved understanding of lactobacilli and their probiotic properties (Claesson *et al.,* 2007; Ventura *et al.,* 2008). Many *Lb. crispatus* strains have been subjected to genome analyses. *Lb. crispatus* ST1, a chicken crop isolate (Ojala *et al.,* 2010), has its genome fully sequenced, while a further nine strains have draft genome sequences available.

Lb. crispatus EM-LC1 was isolated following an extensive screening programme of faecal samples obtained from elderly subjects participating in the ELDERMET project (http://eldermet.ucc.ie). The aim of this screening programme was to identify bacteriocin-producing intestinal bacteria. *Lb. crispatus* EM-LC1 exerted inhibitory activity against *Lb. delbrueckii* subsp. *bulgaricus* LMG6901 *in vitro* (Chapter 6), which on initial phenotypic analysis, appeared to be due to bacteriocin production. However, further characterisation indicated that the antimicrobial agent was heat labile and insensitive to protease (proteinase K, protease, pronase and pepsin) inactivation in the cell-free supernatant. The present study was based on the hypothesis that a genome sequencing approach could be used to identify genes responsible for what initially appeared to be an atypical bacteriocin-like antimicrobial activity exhibited by *Lb. crispatus* EM-LC1

Indeed, the genome sequence of this strain will provide a genomic platform for investigation of *Lb. crispatus* antimicrobial activity and will elucidate the genetic basis for its potential probiotic traits.

3.0 Materials and Methods

3.1 Organism

Lactobacillus crispatus EM-LC1 was originally isolated from a faecal sample obtained from a community-dwelling, elderly male subject (EM367), aged 80 y. This isolate exhibited antimicrobial activity in the cell-free supernatant (CFS) against *Lb. delbrueckii* subsp. *bulgaricus* LMG6901 (Chapter 6). *Lb. crispatus* ST1 is a chicken crop isolate which was obtained from University of Helsinki, Finland. This isolate did not exhibit antimicrobial activity in CFS against *Lb. bulgaricus* LMG6901 (Chapter 6). The genome sequence of this strain is publically available online (Ojala *et al.,* 2010).

3.2 Genomic DNA isolation

EM-LC1 was grown overnight (~18 hours) in modified de Man, Rogosa, and Sharpe (mMRS) broth comprising MRS medium (Difco Laboratories, Detroit, MI, USA) supplemented with 0.05% (w/v) cysteine hydrochloride. DNA was extracted from 15 mL of a fresh overnight culture. Briefly, the culture was sedimented by centrifugation and the resulting cell pellet was resuspended in 5 mL TE buffer (10mM Tris-HCl; 1mM EDTA; pH 8.0) and stored overnight at -20ºC. The cell suspension was thawed, treated with 5 mL of 30 mg mL^{-1} lysozyme and incubated for 60-90 min at 37ºC. Sodium dodecyl sulfate (SDS) was added to a final concentration of 1% before adding 50 µL of a proteinase K solution (100mg/mL) and incubating at 55ºC for 30-60 min, with occasional mixing. The sample was treated with 0.1 volume of 5M sodium chloride followed by 0.1 volume of 5M sodium perchlorate. A further 0.5 volume of phenol/chloroform (25:24:1 phenol:chloroform:isoamyl alcohol) was added and mixed at room temperature on a shaking platform for 30 min. The solution was centrifuged at 3,200 g for 10 min and the aqueous phase was treated with 0.1 volume 3M sodium acetate and 2 volumes of ice cold 96% ethanol was added slowly. DNA (white thread like mass) was spooled out using a glass hook, dipped in 70% ethanol for 30-60 s, air dried to remove residual ethanol, and resuspended in TE buffer (pH 8.0). DNA was applied to a 1% agarose gel and visualized by UV illumination after ethidium bromide staining.

3.3 Genome sequencing and annotation

The genome was sequenced at Macrogen (Seoul, South Korea) on the Illumina platform, generating a paired-end library containing 35,397,530 reads of 101bp. The data were assembled into 54 scaffolds using the *de novo* assembly program Velvet (Zerbino & Birney, 2008). MAUVE was used to reorder scaffolds based on the reference genome of *Lb. crispatus* ST1 (Ojala *et al.,* 2010). tRNA genes were identified using tRNA-scan SE (Lowe & Eddy, 1997). Protein coding regions were predicted using Metagene (Noguchi *et al.,* 2006) and annotation was subsequently performed on the basis of BLASTP (Altschul *et al.,* 1990) analysis against a non-redundant protein database (nr) provided by the National Centre for Biotechnology Information (NCBI) (Wheeler *et al.,* 2007). This automated annotation was then manually inspected in the graphical user interface, Artemis (Rutherford *et al.,* 2000). A functional classification was also applied by using the clusters of orthologous proteins (COG) database (Cut off: E value $<1 \times 10^{-4}$ and 50% identity over at least 50% of both protein sequences) (Tatusov *et al.,* 2001). Metabolic predictions were made by KAAS (KEGG Automatic Annotation Server (Moriya *et al.,* 2007). rRNA loci were detected on the basis of BLASTn searches. Pseudogenes were not determined due to the draft nature of the genome sequence.

Accession numbers: This draft whole genome shotgun project has been deposited in GenBank under the accession no. AXLM00000000. The version described in this paper is the first version, AXLM01000000. The complete genome sequence of *Lb. crispatus* ST1 (accession number NC_014106) (Ojala *et al.,* 2010) was obtained from Genbank (http://www.ncbi.nlm.nih.gov/nuccore/NC_014106.1).

3.4 Genome comparisons

Whole genome nucleotide alignments were generated using BLASTn (blast version 2.2.21). Alignments were then visualised using the Artemis Comparison Tool (ACT) (Carver *et al.,* 2005). Genome sequence alignments were also performed at DNA level using the MUMmer package (Kurtz *et al.,* 2004). Identification of orthologs, paralogs and unique genes was performed following a preliminary step consisting of the comparison of each protein against all other proteins using BLASTP analysis (Altschul *et al.,* 1990) (Cut off: E value $<1 \times 10^{-4}$ and 50% identity over at least 50% of both

protein sequences), then all proteins were clustered into protein families using MCL (graph theory-based Markov clustering algorithm) (van Dongen, 2000).

4.0 Results and Discussion

4.1 General genome features

The *Lb. crispatus* EM-LC1 draft genome sequence consists of 1,862,161 bp with an overall G+C content of 36.9 % (Table 1), a value similar to that of other *Lactobacillus* genomes (Cremonesi *et al.,* 2012; Ojala *et al.,* 2010). All predicted genes, proteins, enzymes and their functions are putative. *In silico* analyses revealed that the genome contains 1,827 protein-encoding genes, representing a coding density of 87.6%, and with an average gene length of 893bp. No rRNA locus was assembled due to exclusion of repetitive sequence from the assembly. We identified forty-five tRNA-encoding genes representing 16 amino acids and twenty seven transposase-encoding genes (Fig. 1).

Functional classification of the predicted genes by Clusters of Orthologous Groups (COGs) of genes (Tatusov *et al.,* 2001) showed that 1383 (75.7%) of predicted protein-encoding genes were homologous to known gene families, including 155 (8.5%) identified as ‘general function prediction only’ and 130 (7.1%) poorly characterised gene functions designated as ‘function unknown’, while 444 (24.3%) do not have any COG association (Fig. 2).

4.2 Comparative genomics

As *Lb. crispatus* ST1 (a chicken crop isolate) is the only strain among the *Lb. crispatus* species with a complete genome sequence available, it was used in a comparison study with *Lb. crispatus* EM-LC1. At a summary statistical level, the genomes of *Lb. crispatus* ST1 and *Lb. crispatus* EM-LC1 are relatively similar (Table 1). *Lb. crispatus* ST1 and *Lb. crispatus* EM-LC1 have the same G+C content of approximately 37%. However, *Lb. crispatus* ST1 has a larger genome size of 2.04 Mbp. In addition, *Lb. crispatus* ST1 has a higher number of coding sequences and rRNA loci but this difference may be a reflection of the draft nature of the genome sequence of *Lb. crispatus* EM-LC1. Further comparative analysis revealed a high degree of synteny between the genomes (Fig. 3). *Lb. crispatus* EM-LC1 contained 135 genes which were

absent in *Lb. crispatus* ST1 at the cut-off value for orthology imposed for their proteins (Table 2). The EM-LC1 specific proteins included those for a zeta/epsilon family toxin/antitoxin system and genes related to bacteriocin (helveticin) production which will be further discussed below. As expected, a large proportion of the unique proteins corresponded to hypothetical proteins.

4.3 Integrated plasmids

Two scaffolds containing two probable integrated plasmids were identified in the genome sequence of EM-LC1, as indicated by the presence of the plasmid-associated genes (Table 3). Scaffold #16, consisted of 25,460 bp (G+C content, 42.9%) and Scaffold #43, consisted of 10,242 bp (G+C content, 37.4%). The latter was found to encode a putative toxin-antitoxin (TA) system of the zeta/epsilon family (Ojala *et al.*, 2010).

4.4 Gastrointestinal-related genes

EM-LC1 has a number of putative genes that have been highlighted (Altermann *et al.*, 2005; Cremonesi *et al.*, 2012; Pridmore *et al.*, 2004) as encoding features likely to contribute to the ability of lactobacilli to colonize and interact with the intestinal mucosa and microbiota. In EM-LC1, five genes encoding mucus-binding proteins and one gene encoding a fibronectin-binding protein were found. These proteins are common to lactobacilli that colonise the gastrointestinal tract and are thought to play an important role in adhesion to the intestinal mucus layer (Azcarate-Peril *et al.*, 2008; Kankainen *et al.*, 2009). In addition, 8 genes encoding S-layer proteins were identified. As the outermost component of the cell wall in several species of *Lactobacillus* (Åvall-Jääskeläinen & Palva, 2005; Beganović *et al.*, 2011; Chen *et al.*, 2007; Konstantinov *et al.*, 2008; Sun *et al.*, 2013), S-layers are considered to protect the cell in hostile environments and aid in the maintenance of cell integrity (Åvall-Jääskeläinen & Palva, 2005). Recently, the S-layer proteins of *Lb. crispatus* were shown to be involved in its adherence to intestinal epithelial cells and may be involved in the competitive exclusion of pathogens (Chen *et al.*, 2007; Sun *et al.*, 2013). The genome of EM-LC1 harbours a gene for a sortase-like protein (Lc367_1071). Sortase enzymes function by anchoring proteins containing the characteristic substrate LPxTG motif to the

peptidoglycan of the cell wall. Indeed, a number of genes in EM-LC1 were annotated as LPxTG motif cell wall anchors (Lc367_1028 and Lc367_1769).

The genome contains a number of predicted proteins assigned to COGs in the carbohydrate transport metabolism category, accounting for approximately 9.1% of the total predicted proteins. *Lb. crispatus* is currently regarded as homofermentative (Charalampopoulos & Rastall, 2009), meaning that sugars can be fermented only via the Embden-Meyerhof-Parnas pathway. The genome sequence encodes a variety of genes associated with carbohydrate utilisation, including 29 putative genes related to the phosphotransferase systems (PTS), by which the sugars are transported into the cytoplasm and phosphorylated. Putative PTS transporters were identified for cellobiose, mannose, sucrose, mannitol, α-glucoside, glucose, glucitol/sorbitol, β-glucoside, and fructose. A number of other genes related to ABC-type sugar transport systems and permeases were also identified.

Intestinal commensal bacteria must also be able to endure a range of physiological stresses. Indeed, the ability of bacteria to respond to stresses such as those encountered during gastric and intestinal transit is key to their survival. The EM-LC1 genome encodes a number of stress resistance proteins including those predicted to confer resistance to heat (Lc367_0124), cold (Lc367_0766), alkali (Lc367_1131 and Lc367_1146) and phage shock proteins (Lc367_1357). In addition, two genes contained conserved domains that can identify them as putatively involved in the general stress responses (Lc367_0067 and Lc367_0710, universal stress protein family, USP). One gene encoding a putative bile salt hydrolase was identified in the genome sequence of EM-LC1 and is likely to be involved in the ability of *Lb. crispatus* to tolerate bile and to survive in the human gut environment.

4.5 Polysaccharide biosynthesis cluster

Many lactobacilli produce extracellular polysaccharides (EPS). Although the physiological role that EPS plays in the bacterial ecology of the commensal LAB still remains uncertain, a number of biologically significant roles, including stress resistance, adhesion, and interaction with the immune system have been attributed to EPS production (Prasanna *et al.*, 2012). A predicted exopolysaccharide (EPS) cluster was identified in the genome sequence of EM-LC1, which was similar to an equivalent

cluster in *Lb. crispatus* ST1 (Ojala *et al.,* 2010). The EM-LC1 EPS cluster spans 15,085 bp and incorporates 16 predicted coding sequences including putative genes for a number of glycosyl transferases, a polysaccharide transporter, EPS biosynthesis proteins and a number of hypothetical proteins (Table 4). The EPS gene cluster exhibits an atypical G+C content of 31.6%. This suggests that acquisition of the *L. crispatus* EPS encoding region may have been by horizontal gene transfer in the intestinal environment and perhaps some particular selective pressure was required to promote acquisition from an outside genus. However, EM-LC1 was found to not produce EPS *in vitro* when grown on Congo red agar, MRS + 10% glucose and MRS + 10% sucrose (data not shown) indicating that the EPS cluster may be non-functional. However, it must be noted that EPS production in lactobacilli is known to be heavily dependent on culture factors such as the type of carbohydrate in the medium (Raftis *et al.,* 2011), variations of which were not tested in this preliminary analysis.

4.6 Prophages, R/M systems and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

Phage and phage remnants are found in the genomes of most LAB and play a prominent role in species-to-species and strain-to-strain variability (Schroeter & Klaenhammer, 2009). Prophage and their remnants can also encode genes directing phenotypes important for host survival and functions. The genome of EM-LC1 contains no complete prophage, similar to *Lb. crispatus* ST1. However, single ORFs with similarities to phage-genes were identified (Lc367_ 0662, Lc367_ 0684).

The genome of *Lb. crispatus* EM-LC1 was also assessed for the presence of phage protection systems. The genome was screened for potential restriction/modification (R/M) systems. R/M systems are associated with the restriction of foreign DNA and are the most common systems used to degrade unmodified phage DNA (Azcarate-Peril *et al.,* 2008; Labrie *et al.,* 2010). The EM-LC1 genome contains a complete type I restriction modification (R/M) system (Lc367_1030, Lc367_1031 and Lc367_1032) and an independent putative restriction endonuclease (Lc367_0032). Type I R/M systems encode three subunits: a subunit for methylase activity (Lc367_1031), a subunit containing the specificity domain (Lc367_1032) and a third subunit which functions as the restriction unit (Lc367_1030). Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated proteins, which provide acquired immunity against

viruses, are absent in the EM-LC1 genome. In contrast, *Lb. crispatus* ST1 was found to encode CRISPR-associated proteins.

4.7 Toxin-antitoxin systems

Toxin-antitoxin (TA) systems are widely distributed on plasmids and chromosomes of prokaryotes, and some often have them in multiple copies (Fozo *et al.,* 2010). In contrast to *Lb. crispatus* ST1, a complete putative TA system of the epsilon/zeta family was encoded in *Lb. crispatus* EM-LC1 (Lc367_1801 and Lc367_1802). The zeta/epsilon module consists of the labile antitoxin protein, epsilon (Lc367_1801), which interferes with the action of the long-living zeta toxin through protein complex formation (Lioy *et al.,* 2012). However, upon antitoxin degradation, the toxin induces cell stasis/death. Zeta toxins target bacterial cell wall synthesis and dependent on the physiological state of bacteria, zeta poisoning can either induce a lytic or bacteriostatic phenotype (Mutschler & Meinhart, 2013). Whilst initially identified as plasmid maintenance systems that ensure stable inheritance, TA systems have also been linked with numerous functions, including growth modulation, genome maintenance, and stress response (Mutschler *et al.,* 2011; Mutschler & Meinhart, 2013).

Another putative TA locus, a toxin of the Txe/YoeB family (Lc367_1479) and an antitoxin of the RelB/DinJ family (Lc367_1478) was also identified in the genome. A similar type TA locus was identified in the genome sequence of *Lb. crispatus* ST1. In addition, a number of individual antitoxin genes of the RelB/DinJ family (Lc367_0449, Lc367_0453 and Lc367_1242) were encoded in the genome which did not have ORFs corresponding to the toxin associated with them as reported previously (Fozo *et al.,* 2010).

4.8 Amino acid biosynthesis and proteolytic activity

Through *de novo* synthesis and inter-conversions, *Lb. crispatus* appears to be able to synthesize 3 of the 20 amino acids (serine, glycine, and cysteine) (Fig. 4 and Fig. 5). Present in the genome is the gene predicted to encode the enzyme L-serine dehydratase (EC. 4.3.1.17) which catalyses the conversion of pyruvate into serine. Serine in turn can be converted into glycine by glycine hydroxymethyltransferase (EC. 2.1.2.1). *Lb. crispatus* is also potentially capable of converting pyruvate to L-cysteine using the enzyme cystine lyase (EC. 4.4.1.8). A high degree of auxotrophy has been found in

other lactobacilli commonly residing in the human gastrointestinal tract, such as *Lb. acidophilus* (Altermann *et al.,* 2005), and *Lb. gasseri* (Azcarate-Peril *et al.*, 2008) and *Lb. johnsonii* (Pridmore *et al.,* 2004). Indeed, *Lb. johnsonii* has been shown to completely lack genes encoding biosynthetic pathways for amino acids. This highlights the dependence this bacterium has on extracellular sources of amino acids that are likely to be present in the intestinal milieu.

Because EM-LC1 is highly dependent on exogenous amino acids for growth, it is therefore not surprising that it possesses a large number of peptidase- and protease-encoding genes to release these from proteinaceous substrates in the GIT. The proteolytic system of LAB usually consists of a cell wall-associated serine proteinase, transport systems specific for di-, tri- and oligopeptides and a multitude of intracellular peptidases (Slattery *et al.,* 2010). *Lb. crispatus* EM-LC1 does not appear to encode the main enzyme required for large polypeptide utilisation, the extracellular protease that is involved in the primary breakdown of proteins. However, *Lb. crispatus* EM-LC1 has uptake systems for peptides, which are the main protein-degradation products. *In silico* analysis of the predicted transporters revealed the presence of a number of ATP-binding cassette (ABC)-type transporters translocating both oligopeptides and amino acids. Once internalised, the peptides are degraded by a variety of peptidases, which have been extensively studied in LAB (Savijoki *et al.,* 2006). *Lb. crispatus* EM-LC1 was shown to encode a number of putative endopeptidases, proline peptidases, aminopeptidases and dipeptidases.

4.9 Bacteriocins

Lactic acid bacteria produce several antimicrobial compounds, including organic acids (lactic and acetic acid), hydrogen peroxide and bacteriocins. Bacteriocins are defined as ribosomally synthesised antimicrobial peptides that are active against other mainly closely-related bacteria (Cotter *et al.,* 2005; Riley and Wertz, 2002). *Lb. crispatus* EM-LC1 exerts bacteriocin-like antimicrobial activity against *Lb. delbrueckii* subsp. *bulgaricus* LMG6901 *in vitro* (Chapter 6), which on phenotypic analysis, appeared heat labile and insensitive to protease inactivation in the cell-free supernatant.

The draft genome sequence of *Lb. crispatus* EM-LC1 identified a number of bacteriocin-type putative genes. EM-LC1 contains three putative helveticin genes with

homology to the helveticin J structural gene from *Lb. helveticus* 481 (Joerger & Klaenhammer, 1990). The predicted amino acid sequences of Lc367_1308, Lc367_1692 and Lc367_1716 have, respectively, approximately 36%, 57% and 77% amino acid identity to helveticin J (Fig. 6). The genomes of *Lb. delbrueckii* subsp. *bulgaricus* ND02 (Sun *et al.,* 2011), *Lb. amylovorus* GRL1112 (Kant *et al.,* 2011), *Lb. gigeriorum* CRBIP 24.85T (Cousin *et al.,* 2012) and *Lb. acidophilus* NCFM (Altermann *et al.,* 2005) also harbour homologs of helveticin, indicating that such homologs are widespread in lactobacilli. It is interesting to note that two homologs of helveticin J are also present in *Lb. crispatus* ST1 (36% and 75% amino acid identity) (Ojala *et al.,* 2010) (Fig. 7). However, *Lb. crispatus* ST1 did not exert the same antimicrobial activity as *Lb. crispatus* EM-LC1 *in vitro* (unpublished data).

The helveticin J bacteriocin reported by Joerger and Klaenhammer in *Lb helveticus* 481 is a large heat labile bacteriocin that inhibits the growth of a small number of *Lactobacillus* species (Joerger & Klaenhammer, 1986). Joerger and Klaenhammer also reported the existence of a second open reading frame, ORF2, that appeared to be co-transcribed with the helveticin J ORF (Joerger & Klaenhammer, 1990). The EM-LC1 helveticin J homologs, Lc367_1692 and Lc367_1716, each have an ORF homologous to ORF2 (Lc367_1693 and Lc367_1715, respectively) associated with it, while Lc367_1308 does not. Indeed Lc367_1693 and Lc367_1715 show strong similarity (72% and 71% amino acid identity) to the helveticin J ORF2 (Fig. 8). Interestingly, the two helveticin J homologs of *Lb. crispatus* ST1 do not have a second ORF associated with them. It has been speculated that ORF2 may encode a protein which binds to helveticin J and facilitates its export from the cell (Fremaux & Klaenhammer, 1994). The absence of ORF2 in *Lb. crispatus* ST1 may be responsible for its lack of antimicrobial activity compared to *Lb. crispatus* EM-LC1.

Analysis of the genome of *Lb. crispatus* EM-LC1 also identified another region containing genes associated with bacteriocin production, the genetic organisation of which was typical of class II bacteriocins (Altermann *et al.,* 2005; Dobson *et al.,* 2007; Forde *et al.,* 2011) (Fig. 9). Class II bacteriocins are small (<10kDa), heat-stable peptides which undergo removal of a leader peptide during export and activation. The bacteriocin-like region identified in EM-LC1 is composed of putative genes that show homology to the predicted regulatory and export systems of the class II bacteriocin,

lactacin B, encoded by *Lb. acidophilus* (Altermann *et al.,* 2005; Dobson *et al.,* 2007). Although genes encoding a sensor histidine kinase and response regulator (Lc367_1534 and Lc367_1533) and transport apparatus comprising accessory proteins (Lc367_1530 and Lc367_1529) and ATP-binding cassette (ABC) transporters (Lc367_1532 and Lc367_1531) were recognised, no genes homologous to known bacteriocin structural peptides were identified. Similar to the lactacin B operon (Altermann *et al.,* 2005; Dobson *et al.,* 2007), the region appeared to be organised into three clusters: a regulation cluster encoding a histidine kinase and a response regulator protein, an export cluster containing the ABC transporters and accessory proteins and a final cluster composed of proteins of unknown functionality. Unlike the lactacin B operon, the *Lb. crispatus* EM-LC1 locus did not possess a putative 'production complex' preceding the regulation and export machinery (Fig. 9). This production complex of *Lb. acidophilus* NCFM contains four unknown proteins, one of which is hypothesised to act as an inducing peptide (Dobson *et al.,* 2007). Further downstream of the regulation and export cluster of EM-LC1, are the genes encoding four hypothetical proteins. Three of these four unknown proteins, Lc367_1526, Lc367_1527 and Lc367_1528 encode a putative double-glycine leader motif of 16, 18 and 19 amino acids, respectively. Interestingly, *Lb. crispatus* ST1 did not encode putative proteins homologous to Lc367_1526, Lc367_1527 and Lc367_1528. The putative protein Lc367_1528 was homologous (74% amino acid identity) to one of the unknown proteins in the lactacin B cluster (LBA1792) of *Lb. acidophilus* NCFM (Altermann *et al.,* 2005; Dobson *et al.,* 2007). The double-glycine motif acts as a proteolytic site for ABC transporter and accessory protein, indicating that these putative genes are likely to encode proteins that are exported from the cell using this ABC transporter complex (Dobson *et al.,* 2007). Whether the bacteriocin-type locus of EM-LC1 is active remains to be seen. Perhaps these genes may be the remains of a class II-like bacteriocin gene cluster. It has been found previously that many LAB can contain remnants of bacteriocin gene clusters on both the chromosome and resident plasmids (Møretrø *et al.,* 2005; Vaughan *et al.,* 2003).

5.0 Conclusion

Several species of the genus *Lactobacillus* are known to produce bacteriocins (Barrett *et al.,* 2007; De Vuyst *et al.,* 2004; Fremaux & Klaenhammer, 1994; Kawai *et al.,* 2000;

Zamfir *et al.,* 1999). However, little is known about the antimicrobial activities of *Lb. crispatus.* Although *Lb. crispatus* is phylogenetically related to *Lb. acidophilus* (Charalampopoulos & Rastall, 2009) a species which is especially known to produce the bacteriocins (Anjum *et al.,* 2013; Zamfir *et al.,* 1999), to our knowledge, only one study has reported the production of a bacteriocin by a *Lb. crispatus* strain (Tahara & Kanatani, 1997). *Lb. crispatus* EM-LC1 is an elderly faecal isolate which exhibited narrow-spectrum antimicrobial activity, against *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901, *in vitro.* Interestingly, this antimicrobial activity is not exhibited by the chicken crop isolate, *Lb. crispatus* ST1 (Ojala *et al.,* 2010). While *in silico* analysis of the draft genome sequence of *Lb. crispatus* EM-LC1 revealed significant similarities to the genome of *Lb. crispatus* ST1, a series of genes specific to EM-LC1 were identified. In particular, genes related to helveticin production were exclusively present in *Lb. crispatus* EM-LC1 as well as a number of hypothetical proteins located in a class II bacteriocin-like locus which may be responsible for the observed antimicrobial activity.

Genome sequencing of EM-LC1 has also revealed a number of other interesting findings. A predicted EPS biosynthetic cluster, a complete type I R/M system, a complete TA system and individual phage-like genes were also identified. As expected a number of genes related to carbohydrate utilisation and genes implicated in adhesion to human intestinal cells were identified and are likely to contribute to the ability of *Lb. crispatus* to colonize intestinal mucosa.

In conclusion, this draft genome sequence has paved the way for the identification of the potentially novel antimicrobial agent produced by *Lb. crispatus* EM-LC1 and has also shed light on some additional genome features. The genome sequence provides a basis for future comparisons with other *Lb. crispatus* faecal isolates. Genomic safety evaluation of this faecal isolate, as previously described in probiotic strains (Wei *et al.,* 2012; Zhang *et al.,* 2012) may also prove worthwhile to identify potential antibiotic resistance or virulence-associated genes which may be of interest in future studies.

Table 1. Major genomic features of *Lb. crispatus* EM-LC1 and *Lb. crispatus* ST1

Strain	***Lb. crispatus* EM-LC1**	***Lb. crispatus* ST1**
Genome size (bp)	1,862,161	2,043,160
G+C content (%)	37	37
Coding genes	1, 827	2,024
Coding density (%)	87.6	88.6
Average gene length (bp)	893	895
rRNA loci	-	4
tRNAs	45	64
Transposase	26	62
CRISPR loci	0	1
Scaffolds	54	1
Status of assembly	draft	finished
Source	human faeces	chicken crop
Genbank accession number	N/A	NC_014106

Table 2. *Lb. crispatus* EM-LC1 specific proteins as determined by comparison to *Lb. crispatus* ST1

Locus_tag (s)	Predicted function
Lc367_0017	hypothetical protein
Lc367_0192	hypothetical protein
Lc367_0238	hypothetical protein
Lc367_0291	deoxyribose-phosphate adolase
Lc367_0297	CoA transferase
Lc367_0298	formyl coA transferase
Lc367_0303	hypothetical protein
Lc367_0348	hypothetical protein
Lc367_0356	hypothetical protein
Lc367_0374	hypothetical protein
Lc367_0382	ammonium transporter
Lc367_0413	hypothetical protein
Lc367_0442	transposase
Lc367_0443	transposase
Lc367_0444	hypothetical protein
Lc367_0446	hypothetical protein
Lc367_0447	hypothetical protein
Lc367_0451	hypothetical protein
Lc367_0454	hypothetical protein
Lc367_0460	hypothetical protein
Lc367_0461	LtrC-like protein
Lc367_0465	glycogen synthase
Lc367_0466	glucan phosphorylase
Lc367_0467	Pullulanase (partial)
Lc367_0474	hypothetical protein
Lc367_0475	hypothetical protein
Lc367_0476	hypothetical protein
Lc367_0477	hypothetical protein
Lc367_0479	hypothetical protein
Lc367_0480	hypothetical protein
Lc367_0481	hypothetical protein
Lc367_0483	hypothetical protein
Lc367_0664	hypothetical protein
Lc367_0666	hypothetical protein
Lc367_0670	hypothetical protein
Lc367_0683	hypothetical protein
Lc367_0685	hypothetical protein
Lc367_0720	hypothetical protein
Lc367_0721	hypothetical protein
Lc367_0722	hypothetical protein
Lc367_0723	hypothetical protein
Lc367_0724	hypothetical protein
Lc367_0957	hypothetical protein
Lc367_0963	hypothetical protein
Lc367_0966	cytidine deaminase
Lc367_1030	type I R/M system, restriction subunit
Lc367_1032	type I R/M system, specificity subunit
Lc367_1039	conserved hypothetical protein
Lc367_1041	sugar-phosphate isomerase
Lc367_1042	acetyltransferase
Lc367_1063	hypothetical protein
Lc367_1064	hypothetical protein
Lc367_1115	transcriptional regulator
Lc367_1213	dihydroxyacetone kinase, phosphotransfer
Lc367_1215	dihydroxyacetone kinase, DhaK subunit
Lc367_1221	CoA-transferase
Lc367_1222	deoxygenase/reductase
Lc367_1223	crotonase/enoyl CoA hydratase
Lc367_1313	hypothetical protein

Lc367_1324	hypothetical protein
Lc367_1327	hypothetical protein
Lc367_1339	predicted ATPase
Lc367_1428	hypothetical protein
Lc367_1429	HesA/MoeB/ThiF family protein
Lc367_1430	hypothetical protein
Lc367_1453	hypothetical protein
Lc367_1465	glycosyl transferase
Lc367_1466	glycosyl transferase
Lc367_1467	hypothetical protein
Lc367_1526	hypothetical protein
Lc367_1527	hypothetical protein
Lc367_1528	hypothetical protein
Lc367_1530	bacteriocin secretion accessory protein
Lc367_1545	conserved hypothetical protein
Lc367_1671	conserved hypothetical protein
Lc367_1673	hypothetical protein
Lc367_1718	hypothetical protein
Lc367_1731	hypothetical protein
Lc367_1732	hypothetical protein
Lc367_1734	hypothetical protein
Lc367_1737	hypothetical protein
Lc367_1738	conserved hypothetical protein
Lc367_1739	hypothetical protein
Lc367_1741	conserved hypothetical protein
Lc367_1746	hypothetical protein
Lc367_1749	ribonucleotide reductase
Lc367_1777	hypothetical protein
Lc367_1780	N-acetylmannosamine-6-phosphate epimerase
Lc367_1782	transcriptional regulator
Lc367_1786	hypothetical protein
Lc367_1789	transcriptional regulator
Lc367_1791	hypothetical protein
Lc367_1795	hypothetical protein
Lc367_1796	conserved hypothetical protein
Lc367_1797	conserved hypothetical protein
Lc367_1798	hypothetical protein
Lc367_1801	antidote of epsilon-zeta postsegregational killing system
Lc367_1820	hypothetical protein
Lc367_1825	conserved hypothetical protein
Lc367_1826	conserved hypothetical protein
Lc367_1827	hypothetical protein
Lc367_1828	conserved hypothetical protein
Lc367_1829	zinc-dependent metallopeptidase
Lc367_1840	membrane protein
Lc367_0455/Lc367_1802	putative ATPase/zeta toxin
Lc367_0456/Lc367_1803	hypothetical protein
Lc367_0457/Lc367_1804	hypothetical protein
Lc367_0463/Lc367_0464	glucose1phosphate adenyltransferase
Lc367_0660/Lc367_0681	hypothetical protein
Lc367_0661/Lc367_0682	hypothetical protein
Lc367_0662/Lc367_0684	prophage DNA packaging protein
Lc367_0663/Lc367_0665	hypothetical protein
Lc367_0667/Lc367_0686	hypothetical protein
Lc367_0668/Lc367_0687	hypothetical protein
Lc367_1693/Lc367_1715	helveticin ORF2
Lc367_1747/Lc367_1748	hypothetical protein
Lc367_0669/Lc367_0688/Lc367_1776	integrase/recombinase
Lc367_0458/Lc367_1805/Lc367_1806/Lc367_1807	nickase

Table 3. Scaffolds containing potential integrated plasmids

Scaffold	Locus Tag	Predicted function	Position	Size (kb)
#16			**453021..478481**	**25.5**
	Lc367_0441	pullulanase		
	Lc367_0442	transposase		
	Lc367_0443	transposase		
	Lc367_0444	hypothetical protein		
	Lc367_0445	resolvase		
	Lc367_0446	hypothetical protein		
	Lc367_0447	hypothetical protein		
	Lc367_0448	hypothetical protein		
	Lc367_0449	addiction molecule antitoxin, RelB/DinJ family		
	Lc367_0450	chromosome partitioning protein, ParA		
	Lc367_0451	hypothetical protein		
	Lc367_0452	replication initiator protein, RepA		
	Lc367_0453	addiction molecule antitoxin, RelB/DinJ family		
	Lc367_0454	hypothetical protein		
	Lc367_0455	putative ATPase		
	Lc367_0456	hypothetical protein		
	Lc367_0457	hypothetical protein		
	Lc367_0458	nickase		
	Lc367_0459	DNA topoisomerase		
	Lc367_0460	hypothetical protein		
	Lc367_0461	LtrC-like protein		
	Lc367_0462	glycogen brancing enzyme		
	Lc367_0463	glucose-1-phosphate adenylyltransferase		
	Lc367_0464	glucose-1-phosphate adenylyltransferase		
	Lc367_0465	glycogen synthase		
	Lc367_0466	glucan phosphorylase		
	Lc367_0467	pullulanase (partial)		
#43			**1834667..1844909**	**10.2**
	Lc367_1795	hypothetical protein		
	Lc367_1796	conserved hypothetical protein		
	Lc367_1797	conserved hypothetical protein		
	Lc367_1798	hypothetical protein		
	Lc367_1799	replication initiator a, n terminal		
	Lc367_1800	conserved hypothetical protein		
	Lc367_1801	antidote of zeta-epsilon postsegregational killing system		
	Lc367_1802	zeta toxin protein		
	Lc367_1803	hypothetical protein		
	Lc367_1804	conserved hypothetical protein		
	Lc367_1805	nickase (partial)		
	Lc367_1806	nickase (partial)		
	Lc367_1807	nickase (partial)		
	Lc367_1808	large conductance mechanosensitive channel		
	Lc367_1809	transposase		

Table 4. Predicted EPS cluster

Locus Tag(s)	Predicted function
Lc367_1462	acetyl transferase
Lc367_1463	polysaccharide transporter, PST family
Lc367_1464	UDP-galactopyranose mutase
Lc367_1465	hypothetical protein
Lc367_1466	hypothetical protein
Lc367_1467	hypothetical protein
Lc367_1468	hypothetical protein
Lc367_1469	glycosyl transferase
Lc367_1470	glycosyl transferase
Lc367_1471	glycosyl transferase
Lc367_1472	glycosyl transferase
Lc367_1473	phospho-glucosyltransferase
Lc367_1474	exopolysaccharide biosynthesis protein
Lc367_1475	exopolysaccharide biosynthesis protein
Lc367_1476	exopolysaccharide biosynthesis protein
Lc367_1477	exopolysaccharide biosynthesis protein

Figure 1. Genome-atlas view of the *Lb. crispatus* EM-LC1 draft genome sequence. From outside to inside: *Lb. crispatus* genes on the forward strand (green); genes on the reverse strand (red); tRNAs (orange); putative integrated plasmids (purple); helveticin genes (black); putative class II bacteriocin gene cluster (turquoise); polysaccharide biosynthesis cluster (yellow); G+C % (light blue - below mean and dark blue – above mean) and GC skew

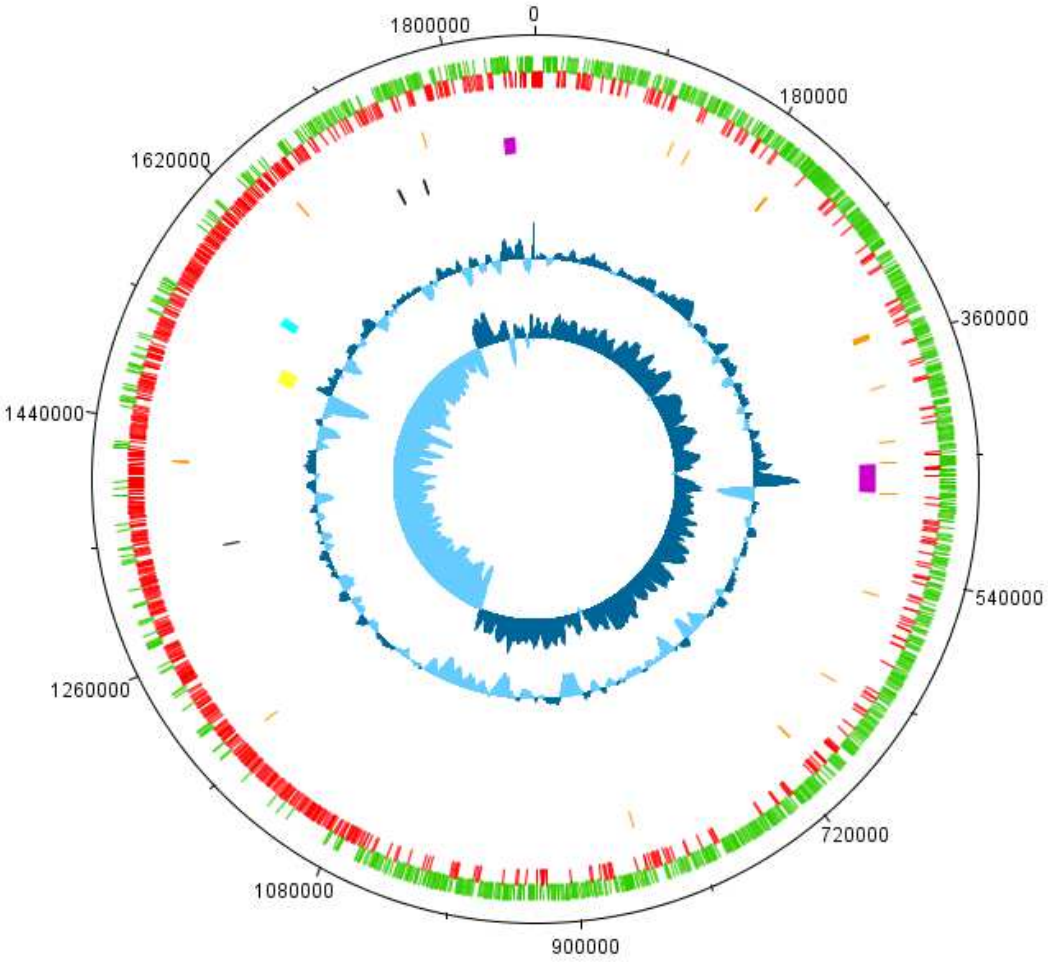


Figure 2. COG distribution in the *Lb. crispatus* EM-LC1 genome. Colours listed as they appear in clockwise rotation from ↓

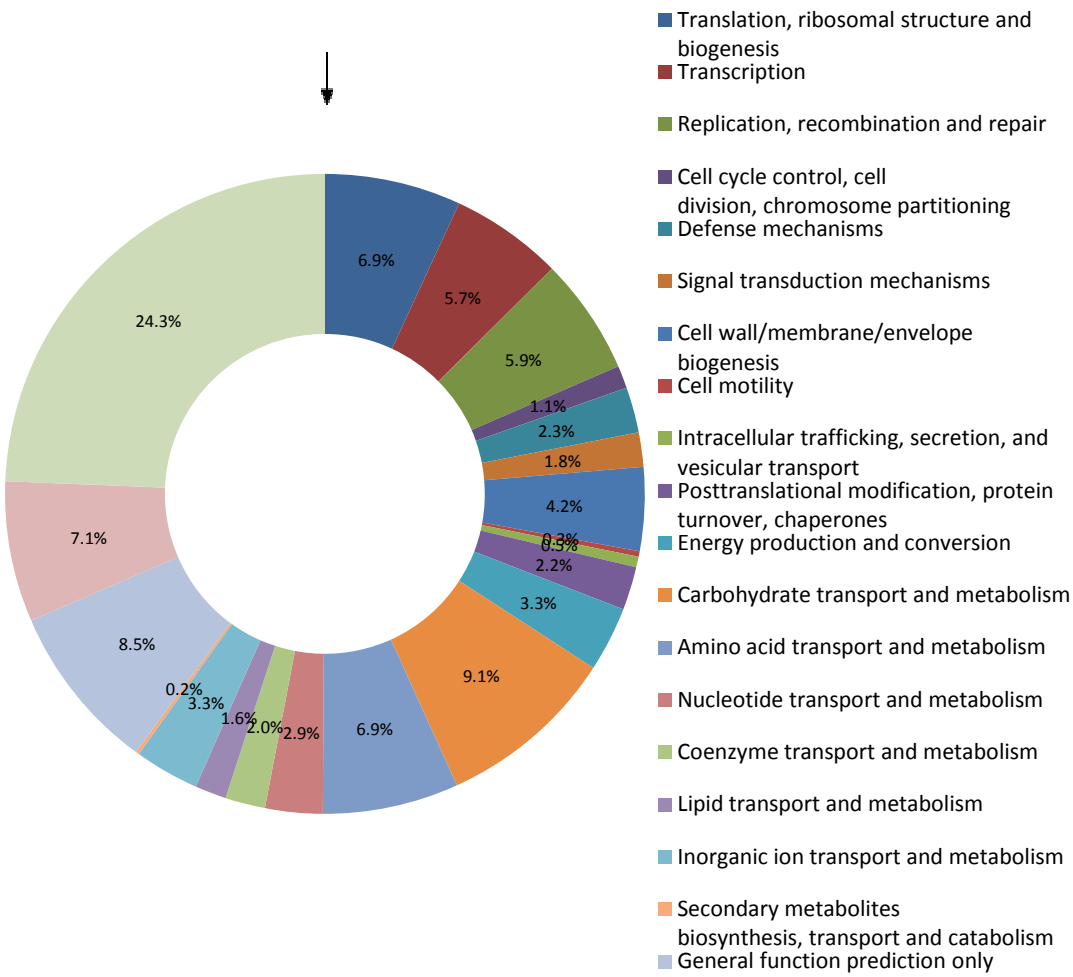


Figure 3. Comparison of the genomes of two *Lb. crispatus* strains. Top panel: MUMMER plot (DNA-DNA) of *Lb. crispatus* ST1 (horizontal axis) and *Lb. crispatus* EM-LC1 (vertical axis) genomes. Red dots represent regions of homology between the genomes and which are in the same orientation. Blue dots represent homology between the genomes in the opposite orientation. Bottom panel: ACT comparison (DNA-DNA) of *Lb. crispatus* ST1 (top) and *Lb. crispatus* EM-LC1 (bottom). Homologous genomic sequences are indicated by red lines. Blue lines indicate homologous genomic sequences in reverse orientation. White indicates regions without homology in the other genome.

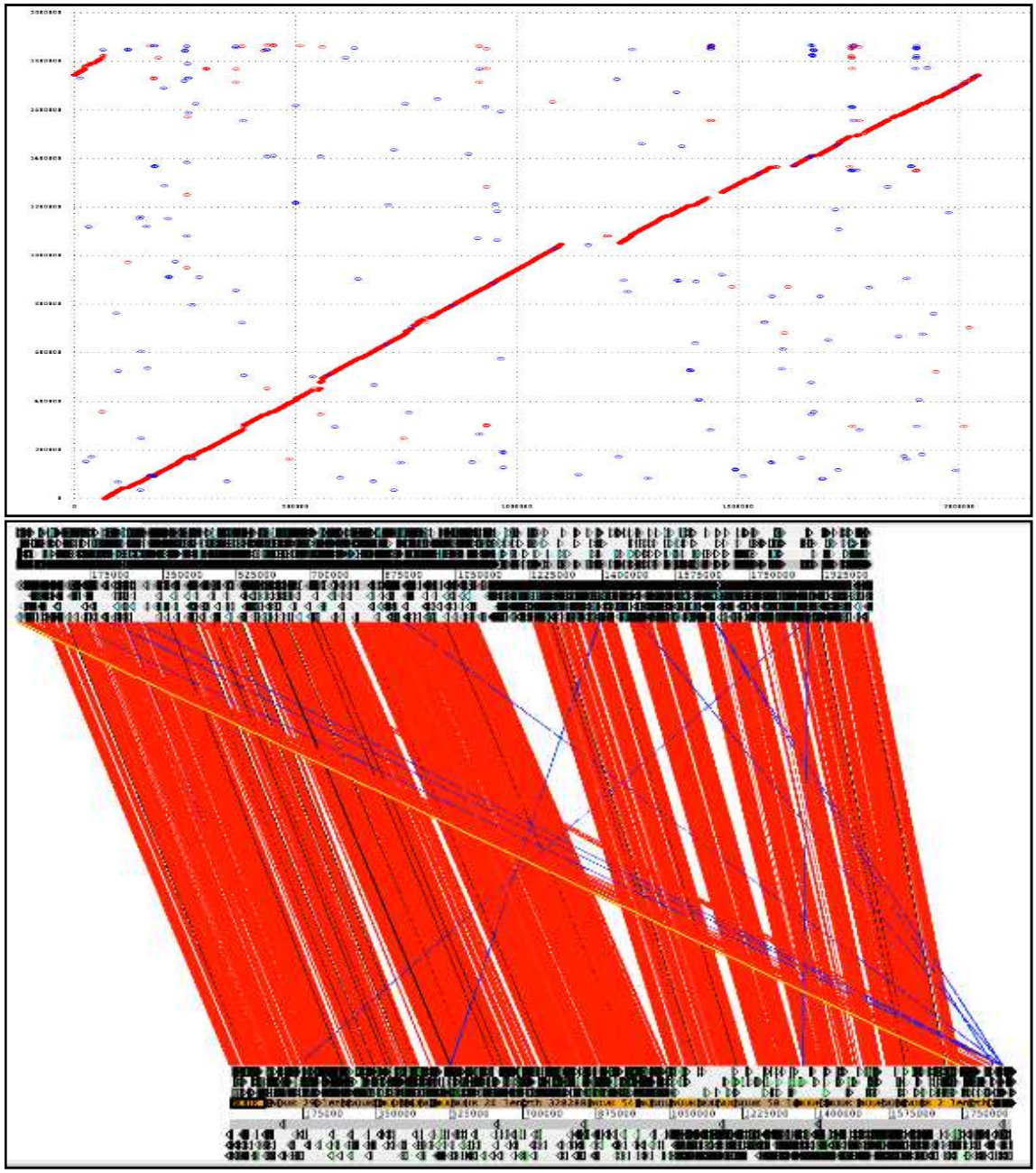


Figure 4. Partial metabolic map of *Lb. crispatus* EM-LC1, showing the predicted interconversions of pyruvate, serine and glycine. Enzyme labels in green boxes represent those for which the corresponding gene was annotated in the genome

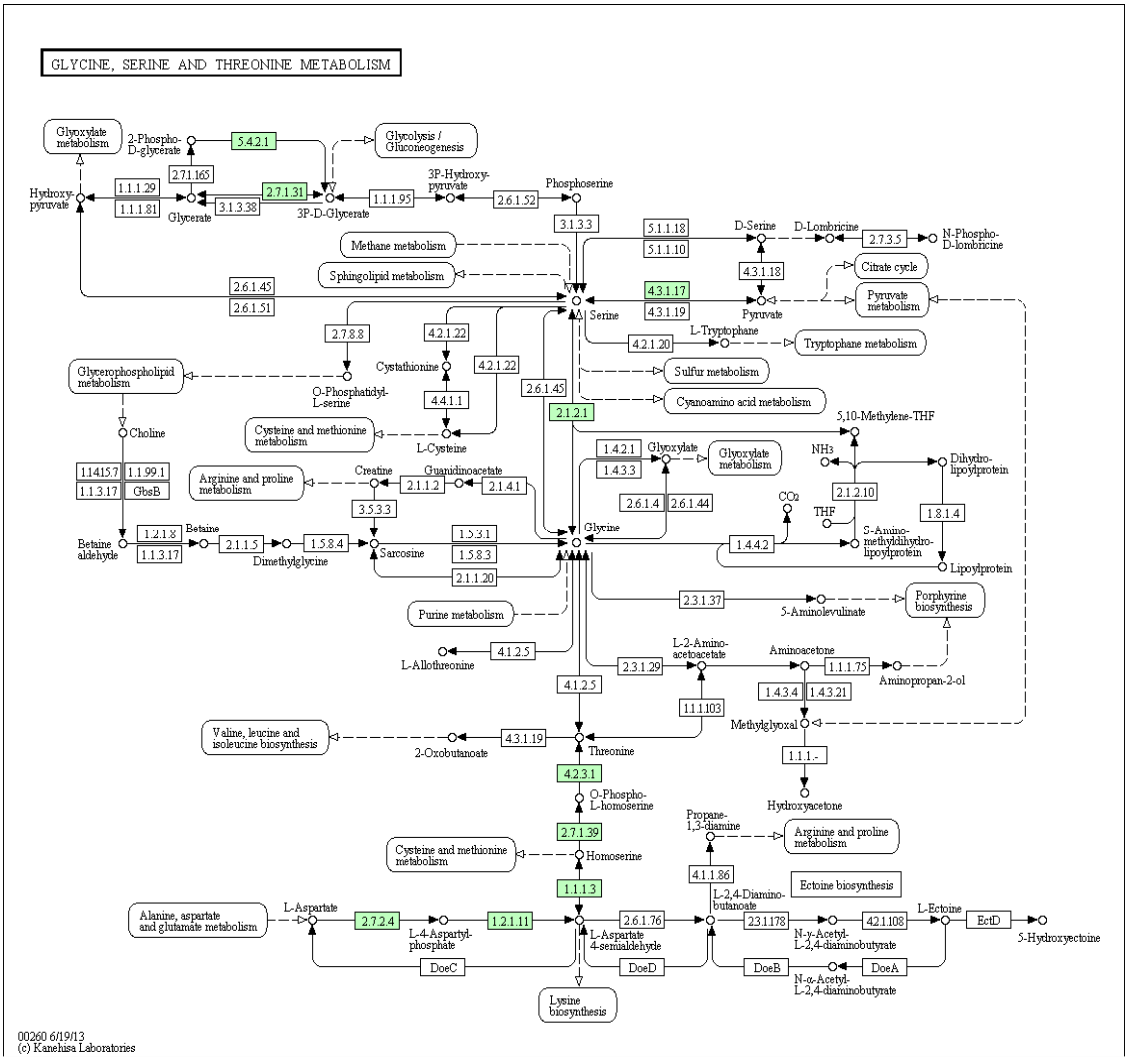


Figure 5. Partial metabolic map of *Lb. crispatus* EM-LC1, showing the predicted interconversion of pyruvate and L-cysteine. Enzyme labels in green boxes represent those for which the corresponding gene was annotated in the genome.

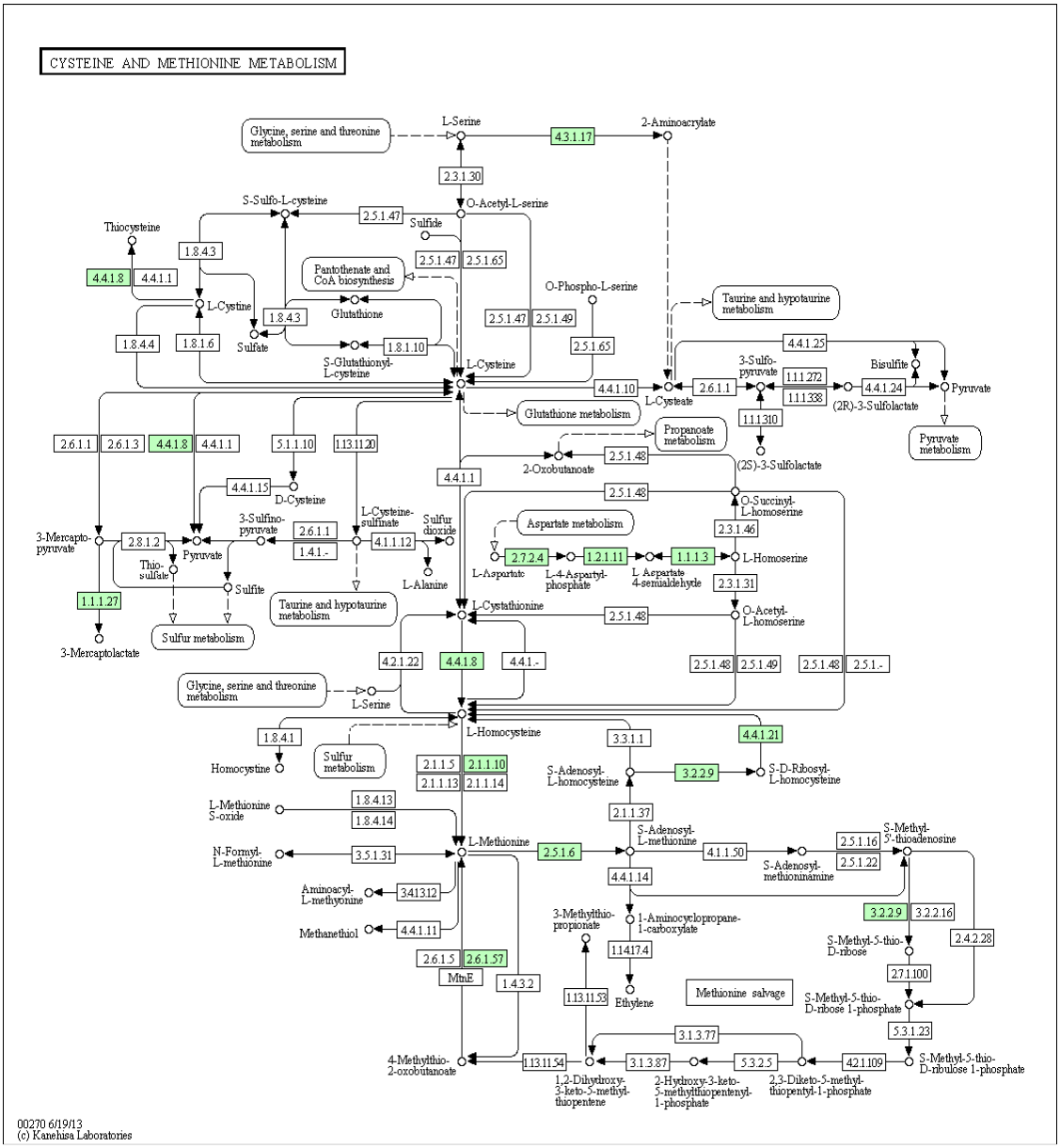


Figure 6. Alignments of EM-LC1 helveticin homologs to helveticin J: A) Em367_1716 (77% aa identity); B) Em367_1692 (57% aa identity); and C) Em367_1308 (36% aa identity)

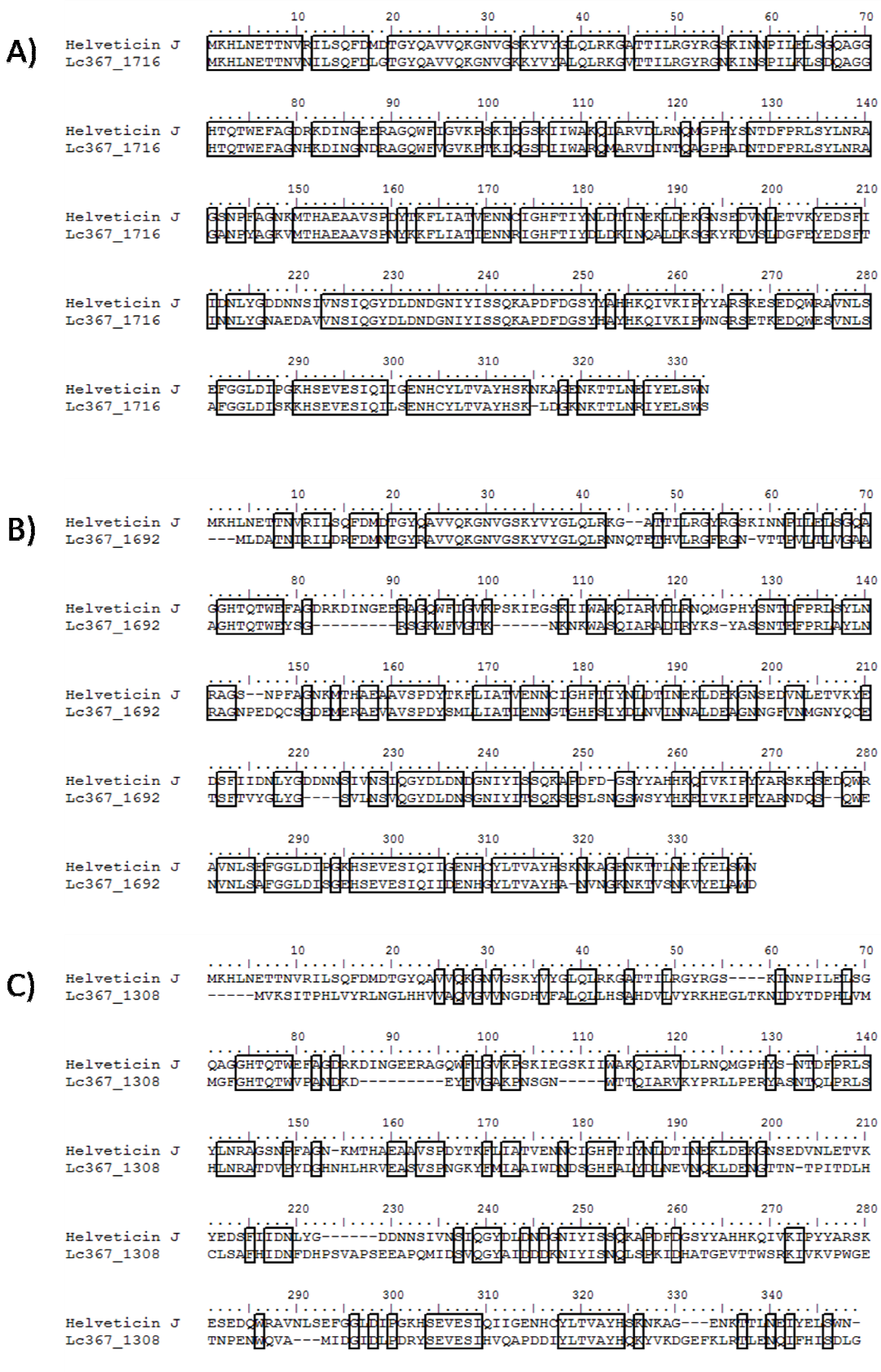


Figure 7. Alignments of *Lb. crispatus* ST1 helveticin homologs to helveticin J: A) LcST1_00011 (75% aa identity); B) LcST1_00534 (36% aa identity)

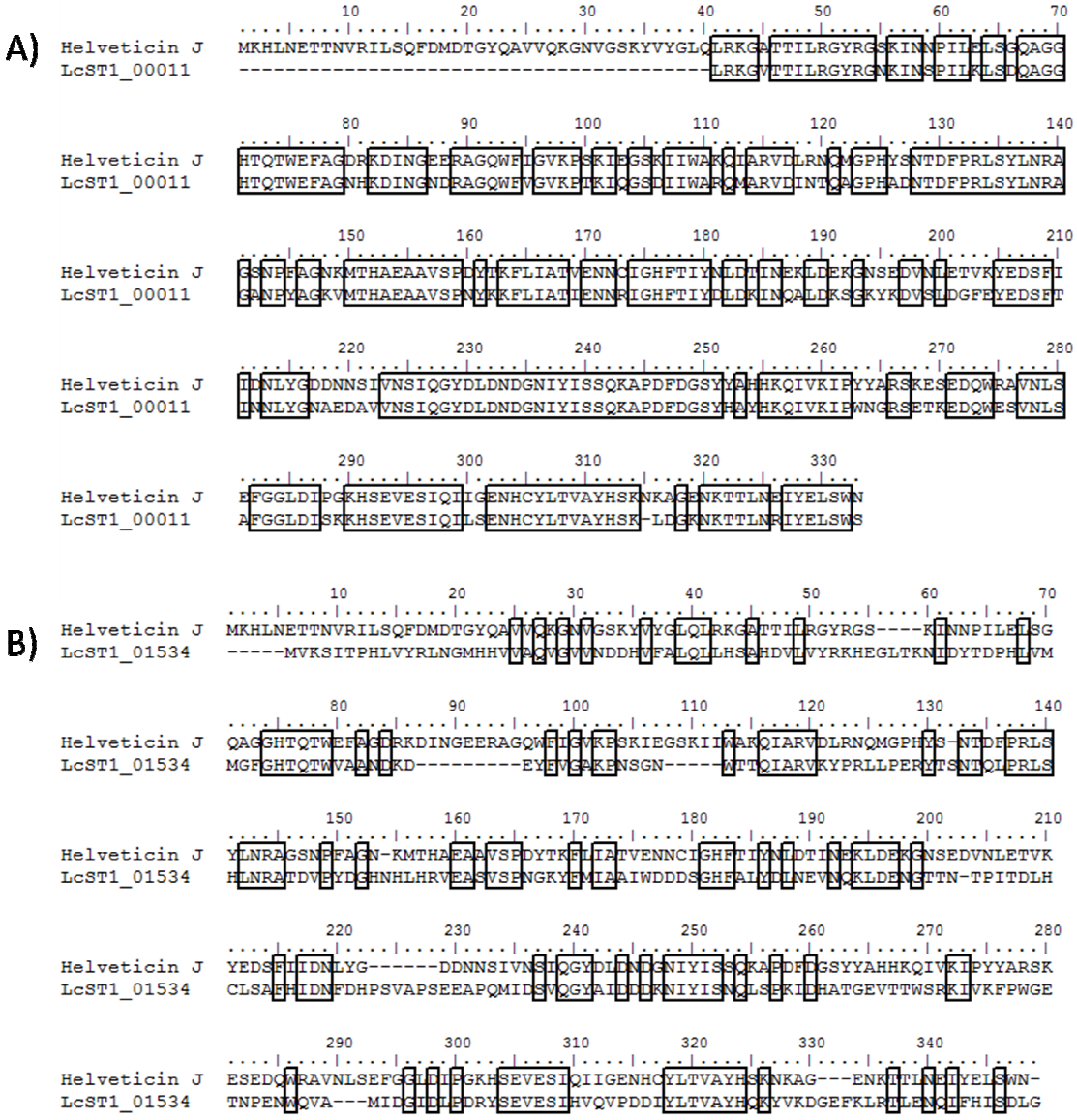


Figure 8. Alignments of EM-LC1 helveticin ORF2 homologs to helveticin J ORF2: A) Em367_1715 (72% aa identity) B) Em367_1693 (71% aa identity)

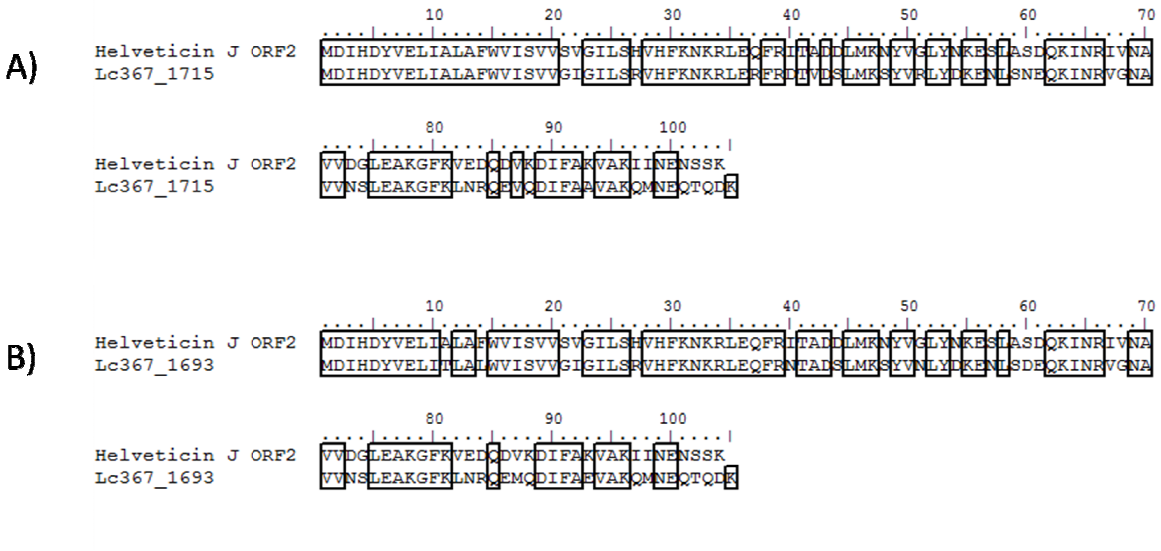
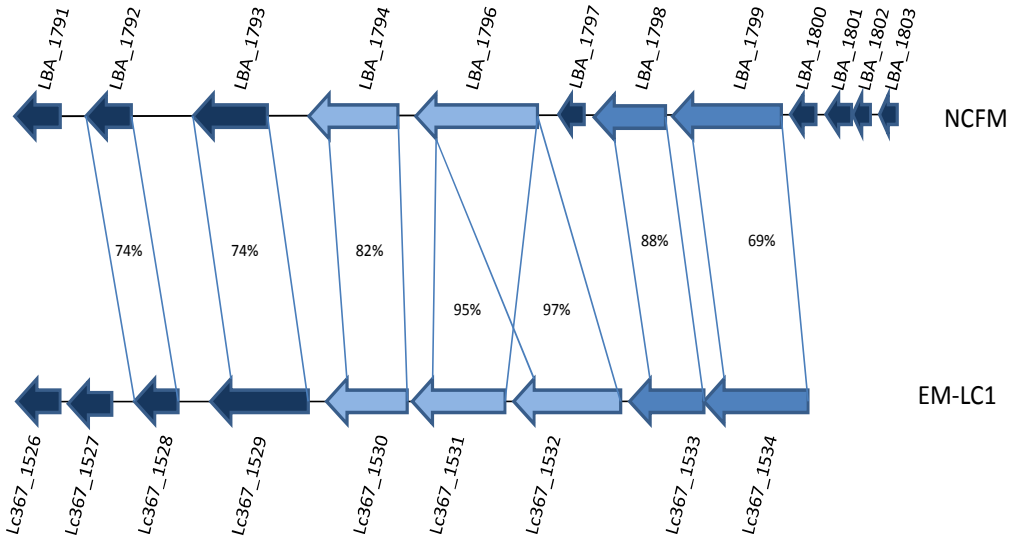


Figure 9. Comparison of the lactacin B cluster of *Lb. acidophilus* NCFM with the locus encoding a putative Class II bacteriocin cluster of *Lb. crispatus* EM-LC1. Figure not drawn to scale. ORFs of unknown function are represented by navy shaded arrows.



Lc367_1534	sensor histidine kinase (405 aa)
Lc367_1533	response regulator (266 aa)
Lc367_1532	ABC-transporter (450 aa)
Lc367_1531	ABC-transporter (261 aa)
Lc367_1530	bacteriocin secretion accessory protein (198 aa)
Lc367_1529	hypothetical protein (434 aa)
Lc367_1528	hypothetical protein (65 aa)
Lc367_1527	hypothetical protein (83 aa)
Lc367_1526	hypothetical protein (64 aa)

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General Discussion

Understanding the role of diet in determining human health and disease is a major objective of modern nutrition. Indeed, diet and nutrition have been recognised for many years as important factors in the promotion and maintenance of good health throughout the entire life course. More recently, the activities of the microbial community that inhabits the intestine and its role in maintaining health has been the subject of extensive research. This complex community has been shown to play a key role in numerous metabolic, physiological, nutritional and immunological processes (Brestoff & Artis, 2013; O'Hara & Shanahan, 2006) and perturbations in the composition or function of the microbiota have been linked to undesirable health conditions including inflammatory and metabolic disorders (Power *et al.*, 2013a; Power *et al.*, 2013b). Recent evidence illustrating the impact of diet on the gut microbiota (Claesson *et al.*, 2012; De Filippo *et al.*, 2010; Wu *et al.*, 2011) highlights this complex community as a potentially new dietary target for the treatment and prevention of disease. Moreover, as this internal ecosystem has been shown to be a rich repository for functional food ingredients (e.g. conjugated linoleic acid and exopolysaccharide), probiotics and even new drugs (Shanahan, 2010), the necessity of incorporating studies of this microbiota into contemporary nutritional research is obvious.

The overall hypothesis of this thesis was that diet and the intestinal microbiota independently and jointly contribute to health in the elderly and that the investigation of habitual dietary patterns and functional microbiota components, can lead to the identification of specific effector mechanisms.

The first section of this thesis investigated the dietary intake of elderly subjects participating in the ELDERMET project (Chapter 3). ELDERMET is an Irish project that aims to determine how diet and lifestyle influence, and are influenced by, the bacteria in your gut, and how this knowledge can be used to promote health in the elderly Irish population. A relatively recent publication by the ELDERMET consortium found that the diversity and composition of the microbiota of elderly subjects is mainly driven by diet, and the microbiota, in turn, may significantly influence inflammatory tone and health status in this vulnerable population group (Claesson *et al.*, 2012). As Poor dietary habits and inadequate nutrient intakes are of particular concern in the elderly (Stanga, 2009), the aim of Chapter 3 was to assess, in great detail, the dietary intakes of Irish community-dwelling elderly individuals in order to identify subgroups

of individuals at nutritional risk. In this chapter, it was demonstrated that community-dwelling, elderly individuals are compliant with a number of food group recommendations. Indeed, fruit and vegetables were consumed regularly among this elderly group indicating an awareness of their positive health effects. However, intakes of dairy produce were inadequate and over-consumption of foods high in fat and/or sugar in the elderly may be contributing to the high rate of overweight/obesity in this population group as well as displacing more nutrient dense foods from the diet. Although malnutrition was not detected among the community-dwelling study participants, sub-optimal intakes of several micronutrients were identified, particularly among males, supporting the hypothesis that there are indeed subgroups within the elderly population at nutritional risk.

Following on from the dietary assessment of community-dwelling elderly, the aim of Chapter 4 was to gain more insight into the role of dietary intake as a determinant of cognitive function in the elderly. Ageing is associated with a loss of cognitive performance and an increased risk of dementia, which, in addition to other age-related chronic disease, is expected to place growing demands on health and long-term care providers (World Health Organisation/National Institute of Health, 2011). The findings in this study indicate a role for diet-related factors in successful brain ageing. It appears that western-type dietary patterns and more specifically high glycaemic index (GI)/glycaemic load (GL) diets are associated with reduced cognitive function in the elderly. Indeed, the results of this study generate a new hypothesis and represent a valuable contribution to a better understanding of the link between dietary factors and cognition in the elderly. Nonetheless, additional prospective investigations, intervention and mechanistic studies are required in order to give a definitive answer as to whether a low GI/GL diet can prevent cognitive decline in the elderly and to elucidate the underlying biological mechanisms.

While previous studies by the ELDERMET consortium (Claesson *et al.*, 2012) have recommended the promotion of healthy ageing by nutritional interventions which target the gut microbiota, the studies described above also emphasise the requirement for interventions to improve dairy consumption and micronutrient intakes and reduce dietary fat intakes among community-dwelling older adults. Moreover, there may also be a rationale for the promotion of a low glycaemic index diet in order to promote

healthier brain ageing. Indeed these findings present both challenges and opportunities for the food industry and healthcare policy makers, to devise age-specific dietary recommendations and to develop attractive, healthy and innovative products and/or fortified meals tailored for the elderly. However, it must be noted that in addition to the assessment of the elderly diet and the nutrition–ageing relationship, successful nutritional policies and dietary interventions for healthy ageing require a lucid understanding of what is driving the elderly to adopt specific diets (Irz *et al.*, 2013). It is necessary to understand their food habits; how they choose their food and how or if they take into account the impact of food on their health (Delaney & McCarthy, 2011) in order to learn what can be done at population or individual level to increase the healthiness of their diets.

While the primary role of diet is to provide enough nutrients to meet metabolic requirements, recently, the focus of scientific investigations has moved to the hypothesis that, beyond meeting nutrition needs, diet may modulate various physiological functions which may play a role in health and disease (Granato *et al.*, 2010). Foods which promote health beyond providing basic nutrition are termed 'functional foods' (Sanders, 1998). Of these foods, probiotics, found mostly in dairy products, can exert positive effects on overall health. The probiotic market is lucrative and fast growing and product innovations are expected to play a major role in increasing the share of the market players. Indeed, Alimentary Health Ltd. is the foundation industry partner of the Alimentary Pharmabiotic Centre based at University College Cork and is focused on the discovery, development and commercialisation of proprietary probiotic and pharmabiotic treatments for gastrointestinal disorders and other inflammatory conditions. With this in mind, a collaborative project with Alimentary Health Ltd. (Chapter 5) was undertaken to test the hypothesis that *Bifidobacterium*-fermented milk products (BFMs) containing 'dead' cells could stimulate levels of IL-10 and TNF-α comparable to those stimulated by BFMs containing 'live' cells. Human peripheral blood mononuclear cells were used as an *in vitro* model in this study. Indeed, characterising how the innate immune system responds to probiotic bacteria *in vitro* may give an indication as to the likely immunomodulatory events that can be triggered following probiotic administration *in vivo*. Depending on the strain and the mode of killing used, BFMs containing dead cells stimulated levels of IL-10 and TNF-α which were comparable to those stimulated by

BFM containing live cells. As well as eliminating shelf life problems, administration of such a product containing non-viable microorganisms could reduce the risk of microbial translocation and infection. Nonetheless, the true *in vivo* picture needs to be fully determined taking into account the contributing roles of other commensal gut bacteria as well as the immunocompetent cells in the gut environment.

In the final section of this thesis, an extensive screening study of the elderly-derived faecal microbiota was carried out in order to examine the prevalence of antimicrobial production by intestinal bacteria and to mine this rich repository of metabolites for novel antimicrobials (Chapter 6). This screening system resulted in the isolation of a number common intestinal species (*Escherichia coli, Lactobacillus salivarius, Enterococcus faecium*) with antimicrobial activity, but also selected for *Streptococcus mutans,* a species not normally detected among the human intestinal microbial community (Kamiya *et al.*, 2005). The repeated isolation of previously characterised bacteriocins in this extensive screening study suggests that the culture-based isolation of antimicrobials is perhaps limited in its potential to isolate novel bacteriocin-producers. One major obstacle with such an approach is that bacteriocin production is an unstable trait and may be influenced by a number of environmental factors (e.g. temperature and growth medium). Indeed, the genes responsible for bacteriocin production are typically tightly regulated and bacteriocin production may go undetected under conditions where the responsible operons are switched off (Kleerebezem, 2004). Furthermore, bacteriocins generally have a narrow spectrum of inhibitory activity and might not be detected using only a low number of indicator strains for screening. Future screening studies may benefit from use of a wider range of indicator organisms, particular genera that dominate the gut microbiota (e.g. *Bacteroides, Clostridium and Ruminococcus* species) (Qin *et al.*, 2010). Alternatively, culture-independent, genomic mining tools, such as BAGEL, may be employed (De Jong *et al.*, 2006) which would circumvent the inherent limitations of these type of culture-based screening methods. However, the annotation of bacteriocin-like genes in the genome of a particular strain is insufficient evidence to infer bacteriocin production and a laboratory based confirmation of the phenotype is also required. This study resulted in the isolation of a *Lactobacillus crispatus* strain (EM-LC1) producing a potentially novel heat labile antimicrobial compound which was resistant to protease activity in the cell-free supernatant. A genome sequencing approach was applied to attempt to identify loci which may be

responsible for what initially appeared to be an atypical bacteriocin-like antimicrobial activity.

Genome sequences of commensal bacteria are fundamentally important in understanding the evolution of bacteria including strategies for adaptation to new niches and overcoming competitors (Ahmed, 2009) as well as having a significant predictive-value which can be exploited to inform and direct research hypotheses. We compared the genome sequence of *Lb. crispatus* EM-LC1 to the publicly available genome sequence of *Lb. crispatus* ST1 (a chicken crop isolate which does not exhibit antimicrobial activity *in vitro*) in order to identify genes which may be responsible for the antimicrobial activity. While the genome sequences were shown to exhibit a high degree of similarity, a number of genes were identified which were unique to *Lb. crispatus* EM-LC1, in particular, genes related to bacteriocin (helveticin) production. This information has provided numerous leads for subsequent bacteriocin purification work which is currently ongoing. As well as paving the way for the identification of the potentially novel antimicrobial agent, the genomic characterization of this commensal intestinal bacterium plays an important role in deepening our understanding of its metabolic capacities (carbohydrate utilisation, etc.) and will enable functional and comparative genomic studies to be carried out in the future.

In conclusion, the work presented in this thesis represents another advance in nutrition/microbiota-based research. Indeed this work has provided a considerable amount of evidence supporting the general hypothesis that the investigation of habitual dietary patterns and functional microbiota components could lead to the identification of the specific mechanisms linking both the diet and the intestinal microbiota with health in the elderly. Research related to the dietary intakes of community-dwelling elderly subjects may be used in the development of future public health interventions to improve diet and health of older adults while the research related to the immunomodulatory effect of ‘dead’ probiotics may be employed to generate commercial outputs in the future. Screening the faecal-derived microbiota resulted in the isolation of a potentially novel bacteriocin-producing strain and the subsequent genome sequence of this strain has highlighted a number of genes which may be responsible for the observed antimicrobial activity. Moreover, the genome sequence

provides a basis for future comparisons with other *Lactobacillus* faecal isolates, helping to expand our knowledge of their relationship with mankind.

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Appendices

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British Journal of Nutrition

Review Article

Intestinal microbiota, diet and health

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Abstract

The human intestine is colonised by 10^{13} to 10^{14} micro-organisms, the vast majority of which belong to the phyla Firmicutes and Bacteroidetes. Although highly stable over time, the composition and activities of the microbiota may be influenced by a number of factors including age, diet and antibiotic treatment. Although perturbations in the composition or functions of the microbiota are linked to inflammatory and metabolic disorders (e.g. inflammatory bowel diseases, irritable bowel syndrome and obesity), it is unclear at this point whether these changes are a symptom of the disease or a contributing factor. A better knowledge of the mechanisms through which changes in microbiota composition (dysbiosis) promote disease states is needed to improve our understanding of the causal relationship between the gut microbiota and disease. While evidence of the preventive and therapeutic effects of probiotic strains on diarrhoeal illness and other intestinal conditions is promising, the exact mechanisms of the beneficial effects are not fully understood. Recent studies have raised the question of whether non-viable probiotic strains can confer health benefits on the host by influencing the immune system. As the potential health effect of these non-viable bacteria depends on whether the mechanism of this effect is dependent on viability, future research needs to consider each probiotic strain on a case-by-case basis. The present review provides a comprehensive, updated overview of the human gut microbiota, the factors influencing its composition and the role of probiotics as a therapeutic modality in the treatment and prevention of diseases and/or restoration of human health.

Key words: **Intestinal microbiota: Diet: Health: Probiotics**

The human intestinal microbiota plays a key role in numerous metabolic, physiological, nutritional and immunological processes[1], and perturbations in the composition of the microbiota influences human health[2]. Much of the early information regarding the intestinal microbiota has come from studies that used culture-dependent techniques, which reveal only a minority of species constituting the microbial population[2,3]. However, the advent of culture-independent, DNA-based analyses has generated data that can be mined for information on the composition and functional properties of this hitherto-uncultured microbiota[2,4,5].

The microbial content of the gastrointestinal tract (GIT) changes along its length, ranging from a narrow diversity and low numbers of microbes in the stomach to a wide diversity and high numbers in the large intestine[6,7] (Fig. 1). The best-studied region of the gut is the distal colon, and in adults, faeces-derived populations have been estimated to consist of 10^{13} to 10^{14} micro-organisms, composed of approximately 1100 prevalent species, with at least 160 such species per individual. In its entirety, the microbiota is estimated to contain 150-fold more genes than the human genome[8]. The majority of bacteria belong either to the phylum Firmicutes (including *Clostridium*, *Enterococcus*, *Lactobacillus* and *Ruminococcus*) or to the phylum Bacteroidetes (including *Bacteroides* and *Prevotella* genera), which constitute over 90 % of the known phylogenetic categories found in the human intestine[8–14]. Although there is huge inter-individual variability in microbial compositions[8,9,12,15], recent work has revealed that a core group of more than fifty taxa can be found in nearly half of the human subjects sampled[8,13]. It has also been suggested that the microbiota of most individuals can be categorised into three predominant variants, or 'enterotypes',

Abbreviations: AAD, antibiotic-associated diarrhoea; CD, Crohn's disease; EPS, exopolysaccharide; GF, germ free; GIT, gastrointestinal tract; HC, healthy controls; IBD, inflammatory bowel diseases; IBS, irritable bowel syndrome; LPS, lipopolysaccharide; UC, ulcerative colitis.

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Probiotics in the Prevention and Treatment of Diseases in Adults and Children

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Metabolic Syndrome and Obesity: Adults

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Abstract

The relatively recent discovery that changes in the composition and metabolic activity of the gut microbiota are associated with obesity and related disorders has led to an explosion of interest in this now distinct research field. In the following chapter, we discuss the current evidence related to how the modulation of gut microbial populations might have beneficial effects with respect to controlling obesity. A number of studies in both animals and humans have shown that the composition of the gut microbiota is significantly altered in obesity and diabetes. Strategies including specific functional foods, probiotics, and prebiotics have the potential to favorably influence host metabolism by targeting the gut microbiota. Indeed, probiotics appear to be a promising approach to alter the host metabolic alterations linked to the changes in the gut microbiota. However, the mechanisms by which probiotics may impact on the development of obesity and metabolic health remain unclear and require further investigation.

The prevalence of obesity and its associated metabolic disorders has increased substantially over recent decades to a point where they have reached epidemic levels worldwide. As such, obesity is a major public health issue and is associated with an increased risk of cardiovascular disease (CVD), type 2 diabetes mellitus (T2D), atherosclerosis, nonalcoholic fatty liver disease (NAFLD), and certain cancers. Obesity is a complex condition that results from an imbalance between energy intake and expenditure and appears to be influenced by a combination of genetic, lifestyle, and environmental factors. In this chapter the current evidence linking gut microbiota with the development of obesity and obesity-related metabolic diseases and the effects of interventions and probiotics, in particular, on the management and prevention of obesity will be discussed.

ARTICLE

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Gut microbiota composition correlates with diet and health in the elderly

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Alterations in intestinal microbiota composition are associated with several chronic conditions, including obesity and inflammatory diseases. The microbiota of older people displays greater inter-individual variation than that of younger adults. Here we show that the faecal microbiota composition from 178 elderly subjects formed groups, correlating with residence location in the community, day-hospital, rehabilitation or in long-term residential care. However, clustering of subjects by diet separated them by the same residence location and microbiota groupings. The separation of microbiota composition significantly correlated with measures of frailty, co-morbidity, nutritional status, markers of inflammation and with metabolites in faecal water. The individual microbiota of people in long-stay care was significantly less diverse than that of community dwellers. Loss of community-associated microbiota correlated with increased frailty. Collectively, the data support a relationship between diet, microbiota and health status, and indicate a role for diet-driven microbiota alterations in varying rates of health decline upon ageing.

The gut microbiota is required for development and for homeostasis in adult life. Compositional changes have been linked with inflammatory and metabolic disorders[1], including inflammatory bowel disease[2,3], irritable bowel syndrome[4,5] and obesity[6] in adults. The composition of the human intestinal microbiota is individual-specific at the level of operational taxonomic units (OTUs) and stable over time in healthy adults[7]. The composition of the intestinal microbiota in older people (>65 years) is extremely variable between individuals[8], and differs from the core microbiota and diversity levels of younger adults[8,9]. A feature of the ageing process is immunosenescence, evidenced by persistent NF-κB-mediated inflammation and loss of naive $CD4^+$ T cells[10]. The microbiota is pivotal for homeostasis in the intestine[11], and chronic activation of the innate and adaptive immune system is linked to immunosenescence[12]. Correlations have previously been made between specific components of the microbiota and pro-inflammatory cytokine levels, but these did not separate young adults from older people[9]. Alterations in the microbiota composition have also been associated with frailty[13], albeit in a small cohort from a single residence location.

Deterioration in dentition, salivary function, digestion and intestinal transit time[14] may affect the intestinal microbiota upon ageing. A controllable environmental factor is diet, which has been shown to influence microbiota composition in animal models, in small-scale human studies[15–20] and over the longer term[21]. However, links between diet, microbiota composition and health in large human cohorts are unclear. To test the hypothesis that variation in the intestinal microbiota of older subjects has an impact on immunosenescence and frailty across the community, we determined the faecal microbiota composition in 178 older people. We also collected dietary intake information, and measured a range of physiological, psychological and immunological parameters. Dietary groupings were associated with separations in the microbiota and health data sets; the healthiest people live in a community setting, eat differently and have a distinct microbiota from those in long-term residential care. Measures of increased inflammation and increased frailty support a diet–microbiota link to these indicators of accelerated ageing, and suggest how dietary adjustments could promote healthier ageing by modulating the gut microbiota.

Microbiota and residence location

We previously identified considerable inter-individual variability in the faecal microbiota composition of 161 older people (≥65 years), including 43 receiving antibiotics[8]. To investigate links between diet, environment, health and microbiota, we analysed 178 subjects, non-antibiotic-treated, for whom we also had dietary information, and stratified by community residence setting: (1) community-dwelling, $n = 83$; (2) attending an out-patient day hospital, $n = 20$; (3) in short-term (<6 weeks) rehabilitation hospital care, $n = 15$; (4) in long-term residential care (long-stay), $n = 60$. The mean subject age was 78 (± 8 s.d.) years, with a range of 64 to 102 years, and all were of Caucasian (Irish) ethnicity. We included 13 young adults with a mean age of 36 (± 6 s.d.) years. We generated 5.4 million sequence reads from 16S rRNA gene V4 amplicons, with an average of 28,099 (± 10,891 s.d.) reads per subject.

UniFrac β-diversity analysis indicates the extent of similarity between microbial communities[22]. UniFrac PCoA (principal co-ordinate) analysis of 47,563 OTUs (grouped at 97% sequence identity) indicated a clear separation between community-dwelling and long-stay subjects using both weighted and un-weighted analysis (Fig. 1a, b). Microbiota from the 13 younger controls clustered with

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Compliance with Irish food-based dietary guidelines in elderly subjects recruited from rehabilitation wards and out-patient clinics in Southern region hospitals (The ELDERMET project)

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The elderly population are an "at-risk" group for under-nutrition which is associated with longer duration in hospitalised care as well as increased prevalence of morbidity and mortality(1). Identifying food consumption patterns and the contribution of the main food groups to nutritional status is essential to identify those at risk of under- or over-nutrition. The aim of this study was to establish the frequency of consumption of the major food groups in two groups of elderly Irish subjects, who participated in the ELDERMET project, and to explore their compliance with the recently revised Irish food based dietary recommendations(2).

Subjects were recruited from rehabilitation wards (*n 62*; aged 65–93 y) and out-patient clinics (*n 45*; aged 67–92 y) in two Southern region hospitals. Dietary data was collected using a validated semi-quantitative, food frequency questionnaire (FFQ). Daily food group consumption was estimated and compared to Irish food-based dietary guidelines.

Over 82% of rehab and out-patient subjects complied with the recommended number of servings for breads, cereals and potatoes. In addition, both groups consumed adequate fruit and vegetables (see Table) with 51.6% of rehab and 53.3% of out-patient subjects meeting recommendations to consume 5+ servings per day. However, poor compliance was evident for dairy-rich foods with only 14.5% of rehab and 4.4% of out-patient subjects meeting recommendations. Compliance with recommendations for low-nutrient dense foods, high in fat and/or sugar for rehab and out-patients was also particularly low in both groups at 6.5% and 13.3%, respectively. In addition, 53.2% of rehab and 66.7% of out-patient subjects were classified as overweight or obese according to Body Mass Index (BMI). This high rate of overweight and obesity is in line with general healthy population trends(3).

	Daily consumption (servings)*		
Food Group	**Recommended**	**Rehab (n = 62)**	**Out-patients (n = 45)**
Cereals, Breads, Potatoes, Rice & Pasta	*Males: 4 Females: 3*	4.5 [4.0, 5.3]	4.5 [3.7, 5.2]
Fruit & Vegetables	*5 +*	5.1 [3.0, 7.4]	5.2 [3.6, 8.3]
Milk, Cheese & yogurt products	*3*	2.0 [1.3, 3.2]	1.4 [0.8, 2.5]
Meat, Fish, Poultry & Alternatives	*2*	2.0 [1.5, 2.8]	1.8 [1.6, 2.5]
Foods High in Fat and/or Sugar	*Sparingly (< 3)*	6.2 [5.0, 10.3]	7.5 [4.3, 9.7]

*median [interquartile range] number of servings per day.

In conclusion, consumption of low nutrient density foods is excessive among both rehabilitation and out-patient elderly groups. Poor compliance with the recommended intakes for dairy-rich foods may have negative implications, particularly for bone health. There is a need to explore the reasons for poor compliance with dietary recommendations among these groups and to devise strategies to address this.

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Gender differences in food intake among Irish community-dwelling elderly subjects: The ELDERMET project

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The proportion of citizens older than 65 years of age in Western populations is increasing. It is therefore important to investigate the fundamental role of diet in the prevention of age-associated chronic disease[(1)]. In addition, gender differences in food consumption and nutrient intakes are well recognised[(2)], and need to be further explored and addressed.

The aim of this study was to establish the frequency of consumption of the major food groups within a cohort of elderly, Irish subjects ($n = 210$; 113 male and 97 female 64–93 y), who participated in the ELDERMET project.

Dietary data was collected using a validated semi-quantitative, 147-item food frequency questionnaire (FFQ). Individual food and beverage items were aggregated into 30 (mutually exclusive) food groups. Median daily serving intake was calculated and statistical analysis was conducted using PASW® (version 18.0) for two age categories, 64–75 yrs and ≥ 76 yrs.

Females (64–75y) consumed significantly ($P<0.05$) more unrefined wholegrain cereals, dietary supplements, salad dressings, probiotic yoghurts, fresh soups, fish, hot beverages and fruit than males. Males of the same age range consumed significantly ($P<0.05$) more processed soups, meat products, soft drinks, alcohol, meat and refined cereals (see table). Cross-gender, food group variance among the more senior group ($n = 102$) was limited to alcohol intake; with males consuming significantly more than females ($P<0.05$). In addition, females from both age groups showed higher compliance rates with the recently revised Irish dietary guidelines for adults aged >51 y[(3)].

	Median Daily Serving [interquartile range]		
Food Group[b]	**Male**	**Female**	***P*[a]**
Refined cereals	0.00 [0.00, 0.03]	0.00 [0.00, 0.01]	*0.038*
Unrefined wholegrain cereals	0.65 [0.01, 1.00]	1.00 [0.71, 1.03]	*0.002*
Breads, Rolls, etc	2.33 [1.65, 4.04]	2.04 [1.15, 2.98]	*0.122*
Potatoes, boiled, mashed, etc.	0.94 [0.61, 1.03]	1.00 [0.62, 1.04]	*0.476*
Fruit	2.11 [1.29, 3.31]	2.95 [1.49, 4.54]	*0.049*
Vegetables	3.07 [1.93, 4.35]	2.90 [2.18, 5.39]	*0.171*
Salad dressings	0.03 [0.00, 0.28]	0.28 [0.03, 0.30]	*0.008*
Fresh soups	0.03 [0.03, 0.00]	0.15 [0.01, 0.29]	*0.032*
Processed soups	0.03 [0.00, 0.17]	0.00 [0.00, 0.03]	*0.001*
Meat	0.60 [0.45, 0.77]	0.45 [0.21, 0.71]	*0.016*
Meat products	0.15 [0.03, 0.26]	0.05 [0.01, 0.15]	*0.005*
Chicken/Poultry	0.14 [0.03, 0.29]	0.14 [0.14, 0.29]	*0.085*
Fish	0.17 [0.05, 0.29]	0.24 [0.15, 0.44]	*0.046*
Eggs	0.29 [0.08, 0.71]	0.29 [0.15, 0.30]	*0.729*
Probiotic yoghurts	0.00 [0.00, 0.61]	0.29 [0.00, 1.00]	*0.010*
Dietary supplements	0.00 [0.00, 1.00]	1.00 [0.00, 2.00]	*0.006*
Alcoholic drinks	0.31 [0.01, 1.14]	0.16 [0.01, 0.40]	*0.009*
Soft drinks/ sweetened drinks	0.02 [0.00, 0.26]	0.00 [0.00, 0.03]	*0.009*
Hot beverages	4.00 [2.31, 5.75]	4.54 [3.50, 6.03]	*0.047*

[a]P is the significance of the gender difference by the Mann Whitney U Test. A P value of <0.05 was considered as statistically significant. [b]Other food groups analysed incl.chips, pasta/rice, fish products, chicken products, butter, spreads, high-fat dairy, low-fat dairy, dessets/sweets, savoury snacks and ready meals for which no significant differences ($P<0.05$) existed between groups.

In conclusion, gender differences in food intakes were detected among older Irish adults, especially among males aged 64–75 yrs. Identification of causes related to these differences could influence public health interventions to improve diet and health of the older adults.

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Fish-oil consumption is inversely correlated with depression and cognition decline in healthy Irish elderly adults

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Epidemiological studies investigating associations between diet and cognitive function suggest a protective role for fish and fish-oil consumption in cognitive decline(1) and depression(2), particularly among elderly populations. As longevity increases and elderly subjects become an increasing proportion of populations worldwide, it is important to establish dietary components associated with healthy brain aging.

The aim of this study was to evaluate fish and fish-oil consumption in a sample of healthy, community-dwelling Irish adults and to explore the association between fish and fish oil consumption, and cognitive function and depression score.

Fish and fish product intakes, cognitive function and depressive symptoms were assessed among community-dwelling, elderly adults ($n = 172$; age 64–93 yrs) who participated in the ELDERMET project. Fish and fish product consumption was evaluated using a 147-item, validated, semi-quantitative Food Frequency Questionnaire, where fish intakes were categorized into the following groups; processed/fried fish, white fish, oily fish, shellfish and fish-oil supplements. The Mini Mental State Exam (MMSE) and Geriatric Depression Scale (GDS) were used to determine cognitive function and depressive symptoms, respectively.

Among the fish and fish-products assessed, white fish was consumed most frequently, by 84% of the group, with 72% of consumers complying with recommended intakes of one or more servings of white fish/wk. Seventy-six percent of the group consumed oily fish with 64% complying with recommendations. Shellfish consumption was lowest at 31%. Consumption and compliance to recommended intakes was highest among females for all fish and fish-products, except for processed/fried fish consumption, which was higher among males. Fish-oils were consumed by 19% of the group, of which 55% were female.

No significant correlation was observed between white fish consumption and GDS (Pearson correlation coefficient $r = -0.007$; $P = 0.97$) and MMSE ($r = 0.007$; $P = 0.928$). Similarly for oily fish, there was no association between GDS ($r = -0.072$; $P = 0.427$) and MMSE> ($r = 0.052$; $P = 0.497$), indicating no positive effect of either white or oily fish consumption on improved depressive symptoms or cognitive function. However, an inverse association was found between fish-oil supplement consumption and GDS ($r = -0.229$; $P = 0.010$); suggesting an association between reduced depressive symptoms and increased fish-oil consumption. In addition, a trend for association between fish-oil consumption with MMSE ($r = 0.145$; $P = 0.057$) was also observed, whereby increasing fish-oil consumption was associated with a trend for improved cognitive function.

In conclusion, fish consumption was comparable with a nationally representative sample of healthy, elderly Irish individuals(3). However gender differences indicate better compliance with guidelines among females. Further studies investigating the effect of fish-oil consumption on cognitive function and depressive symptoms in elderly groups are warranted. Fish consumption should be promoted in the elderly as it may prevent brain ageing processes and reduce cognitive decline.

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Screening the elderly gut microbiota for potential novel probiotics

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The microbial community that inhabits the large intestine plays an important role in maintaining intestinal health. Many species that make up the gut microbiota produce antimicrobial compounds, including bacteriocins. The production of bacteriocins can be considered a probiotic trait in that it may confer a competitive advantage to producing organisms over other intestinal microbes, possibly aiding survival within the intestine. Due to the increasing prevalence of antibiotic resistance among pathogens including those responsible for hospital acquired infections such a methicillin-resistant *Staphylococcus aureus* (MRSA), there is need to find novel antimicrobial compounds to combat these microorganisms. Hence, the objective of this study was to screen the anaerobic gut microbiota of the elderly for the presence of novel bacteriocin producers which may have potential as probiotics for the elderly. Faecal samples, obtained from 42 elderly subjects participating in the ELDERMET study, were homogenised, serially diluted and spread-plated on to a number of different growth media for the isolation of 1) total anaerobes; 2) non-spore forming anaerobes; 3) Gram negative anaerobes; 4) total *Enterobacteriaceae* and 5) *Bacteroides* sp. Selected colonies were screened for inhibitory substances by the deferred antagonism plate test against four target organisms, *Lb. bulgaricus*, *E. coli* K-12, *Bifidobacterium breve,* and *Cronobacter sakazakii.*16S rRNA gene sequencing was used to identify bacterial isolates showing zones of inhibition. Molecular fingerprinting using pulse field gel electrophoresis (PFGE) was used to genetically distinguish bacterial isolates. Screening with the indicator organisms *Lb. bulgaricus* and *E. coli* K-12 resulted in the isolation of 16 genetically distinct strains producing bacteriocin-like substances. These included *Lb. salivarius, Lb. gasseri*, *Lb. crispatus*, *S. mutans*, *E. faecium*, and *E. coli.* Two of the strains, *Lb. salivarius* and *E. faecium* inhibited growth of the gastrointestinal pathogen *L. monocytogenes* using the well diffusion method. Further work will characterise these isolates and investigate their potential as probiotics.

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Title: Compliance with Irish food based dietary guidelines in elderly subjects recruited from rehabilitation wards and out-patient clinics in Southern region hospitals (The ELDERMET project)

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Aims: Western populations are ageing, therefore it important to exploit the fact that diet plays a fundamental role in the prevention of age-associated chronic disease(1). Identifying food consumption patterns and the contribution of the main food groups is essential to relate diet to nutritional status, and to identify those at risk of under- or over-nutrition. The aim of this study was to establish the frequency of consumption of the major food groups within two groups of elderly, Irish subjects, who participated in the ELDERMET project and to explore their compliance with Irish food based dietary recommendations.

Methods: Subjects were recruited from rehabilitation wards (*n 58*; aged 65–93 yr) and out-patient clinics (*n 34*; aged 70–92yr) in two Southern region hospitals. Dietary data was collected using a validated semi-quantitative, food frequency questionnaire (FFQ). Daily food group consumption was estimated and compared to Irish food based dietary guidelines.

Results:

		Daily Consumption (servings)*	
Food Group	**Recommended Daily Servings**	**Rehab (n=58)**	**Out-patients (n=34)**
Breads, Cereals & Potatoes	6+	4.4	4.7
Fruit & Vegetables	5+	5.6	6.2
Milk, Cheese & Yoghurt	3	2	1.1
Meat, Fish & Poultry & Alternatives	2	1.9	1.8
Foods high in fat and/or sugar	Sparingly (<3)	6.7	8

*median number of servings per day

Only 12% and 15% of rehab and out-patient subjects met with the recommended number of servings for breads, cereals and potatoes. Poor compliance was also evident for dairy-rich foods with only 19% and 12% of rehab and out-patient subjects meeting recommendations. Both groups consumed adequate fruit and vegetables. Compliance with recommendations for low-nutrient dense foods, high in fat and/or sugar was particularly low in both groups at 3.5% and 12% respectively among rehab and out-patients. In addition, 53% of rehab and 59% of out-patient subjects were classified as overweight or obese according to Body Mass Index (BMI). This high rate of overweight and obesity is in line with general healthy population trends(2).

Discussion: In conclusion, we found high consumption of low nutrient density foods in both rehabilitation and out-patient elderly groups.Compliance with intake guidelines for other food groups including breads and cereals, and dairy-rich foods was poor which may have negative health implications. There is a need to explore the reasons for poor compliance with dietary recommendations in these groups and devise strategies to address this.

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Joint Irish Section and American Society for Nutrition Meeting, 15–17 June 2011, 70th anniversary: 'Vitamins in early development and healthy ageing: impact on infectious and chronic disease'

Consumption of fruit and vegetables among elderly Irish people in long-term institutionalised care: the ELDERMET project

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Fruit and vegetables are an important source of vitamins and minerals such as vitamin C, folate, K and β-carotene [(1)]. High fruit and vegetable consumption may reduce the risk of several chronic diseases, including cancers, CVD, hypertension and stroke[(2)]. The benefits of fruit and vegetable consumption continue into old age. In fact, with advancing age the requirements for some antioxidants may be increased[(3)]. The Irish food-based dietary guidelines therefore recommends consumption of at least five portions of fruit and vegetables daily. The aim of this study was to establish the frequency of consumption of fruit and vegetables within a sample of institutionalised elderly, Irish subjects (*n* 99; twenty-six men and seventy-three women; aged 66–104 years), who participated in the ELDERMET project.

Dietary data was collected using a validated semi-quantitative, 147-item FFQ. Fruit and vegetables that contributed to the recommended five portions a day included all fresh, frozen and canned fruit and vegetables, pure fruit drinks, dried fruit, vegetable soups, tomato-based pasta sauces, baked beans and other pulses. Potatoes and nuts were excluded. In order to estimate the number of servings of fruit and vegetables consumed on a daily basis, frequency of consumption was converted to a single daily serving. The number of servings of fruit and vegetables was then calculated by adding the daily value for each relevant fruit/vegetable item. Statistical analysis was conducted using PASW™ (version 18.0).

Overall, fruit and vegetable intake was below the recommended 5+ servings (see Table). The most commonly consumed vegetables were carrots, parsnips/turnips and cabbage, while the most commonly consumed fruit items were pure fruit drinks, bananas and apples. A large proportion of subjects (71.7%) did not meet recommendations to consume 5+ servings of fruit and vegetables daily. Almost half (46.5%) of these subjects consumed less than three servings a day. Percentage compliance with recommendations for other food groups was also poor. Over all, one-fifth (21.2%) of subjects consumed dietary supplements (including vitamin and mineral supplements) on a daily basis that may contribute to vitamin and mineral intakes.

	Daily consumption (servings)*	SD
Total fruits	1.3	1.8
Total vegetables	2.4	1.3
Total fruit and vegetables	3.7	2.6

*Median number of servings per day.

In conclusion, fruit and vegetable intakes are low in this elderly cohort compared with current dietary recommendations. As a consequence, a large proportion of this population may be at risk of inadequate intakes of important vitamins and minerals. There is a need to explore the reasons for poor compliance with consumption recommendations and devise strategies to address this.

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University College Cork, Ireland, 31st March – 1st April 2011.

Compliance with Irish food based dietary guidelines in community dwelling elderly subjects (The ELDERMET project)

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Western populations are ageing, so it is important to exploit the fact that diet plays a fundamental role in the prevention of age-associated chronic diseases, including obesity, diabetes, heart disease and osteoporosis. Identifying food consumption patterns and the contribution of the main food groups is essential to relate diet to nutritional status, and to identify those at risk of under- or over-nutrition. The aim of this study was to establish the frequency of consumption of the major food groups within a sample of community-dwelling, elderly, Irish subjects (*n* 93; aged 64-93yr), who participated in the ELDERMET project, and estimate their compliance with Irish food based dietary recommendations. Dietary data was collected using a validated semi-quantitative, 147-item food frequency questionnaire (FFQ). Daily food group consumption was estimated and compared to Irish food based dietary guidelines. Less than one fifth of subjects (18.3%) met with recommendations to consume 6+ servings of breads, cereals and potato products per day. Only 9.7% of subjects consumed the recommended 3 servings of dairy products per day. Furthermore, over one third (34.4%) of subjects did not consume milk. A large proportion of subjects (80.6%) consumed >3 servings of foods high in fat and/or sugar, while only 19.4% of subjects met with recommendations to consume these foods sparingly (<3 servings/d). 34.4% of subjects consumed the recommended 2 servings of meat, fish, poultry and alternatives, while 81.7% of subjects consumed fish at least once a week. Over half of subjects (63.4%) complied with recommendations to consume 5+ servings of fruit and vegetables/day. A high rate of overweight/obesity (64.5%) was found among subjects when Body Mass Index (BMI ≥25) was used as an indicator. In conclusion, high consumption of low nutrient density foods is prominent in this group with poor compliance to intake guidelines for other food groups including dairy, breads and cereals. There is a need to explore the reasons for poor compliance with dietary recommendations in this group and to devise strategies to address this.

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